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A PHOTOELECTRIC METHOD FOR THE DETERMINATION OF ERYTHROCYTE OSMOTIC FRAGILITY*

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When erythrocytes are subjected to increasingly hypotonic solutions of a salt, hemolysis of the cells occurs in the lower concentrations. This test for the erythrocyte osmotic fragility in hypotonic solutions is used clinically as an aid in the diagnosis of several disease entities. In certain diseases, such as pernicious anemia, obstructive jaundice, pneumonia, sickle cell anemia, Mediterranean anemia, and others, the osmotic resistance of the patient's red blood cells is increased (decreased fragility). An increased fragility is closely correlated with the presence of spheroidal microcytes, but not absolutely related.[1]

The process of hemolysis probably involves the thickening of the erythrocyte and a decrease in its diameter. In some diseases the red cell diameter is already decreased and the thickness is already above normal. These cells are, therefore, more easily hemolyzed in hypotonic solutions. The point of beginning hemolysis is normally between 0.48 and 0.44 percent saline, and all of the cells are not hemolyzed until an even lower concentration of 0.36 to 0.32 percent saline is attained.[1]

In congenital hemolytic jaundice the red blood cell diameter is decreased and the red blood cells are thicker so they are probably nearer to the hemolyzing point than normal erythrocytes which must become more globular before lysing.[2] The increased fragility, therefore, is due to the spherical shape of the cells because it requires very little fluid to bring them to the form in which their membrane is stretched to the bursting point.

Many methods have been described for the determination of the resistance of erythrocytes to hemolysis.

In 1867 Duncan Johann reported increased fragility in salt solutions of the red blood cells of patients with chlorosis, and in 1873, Malassez observed that cells from different individuals became hemolyzed at different time intervals in the diluting fluid used. Malassez later described quantitative determinations in which he made red blood cell counts at different time intervals to find the number of non-hemolyzed red cells present in the solution. Urcelay established a curve based on the number of red blood cells present at different time intervals and concluded that

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there were two groups of cells present in normal blood, a smaller group of more easily hemolyzed cells and a larger one of more resistant cells.[3]

In 1887, Hamburger devised the test that is most widely used today. Though it has been modified in its details, in essence it has remained the same. In the original paper, defibrinated blood was used; in later papers both oxalated and heparinized blood has been suggested. The amount of blood and of the hypotonic solutions specified has also differed with the author. Washed red blood cells have been suggested by some, while others recommend only whole blood on the premise that washing alters the osmotic resistance.[3] Both Na_2SO_4 and NaCl have been used as the hypotonic solutions in descriptions of the laboratory test. The determination of beginning and end hemolysis, and the reporting of the results have differed in the modifications since 1887, but in the long run, the test is as Hamburger reported it: an interpretation of the erythrocyte osmotic fragility as determined visually by discoloration of the supernatant fluid by hemoglobin released through hemolysis in hypotonic solutions. These salt solutions vary in concentration from 0.30 to 0.60 percent saline.

Many methods have been described at various times for the detection of erythrocyte osmotic fragility measuring either the time required for hemolysis or the hypotonicity of the solution. The counting of the red blood cells remaining after a certain amount of time has elapsed to allow for hemolysis in three different dilutions of salt is one of these methods.[3] The cell count may also be done at different time intervals on the same salt concentration.[3] Another method which is more quantitative, called for centrifugation and measurement of the residue of unhemolyzed cells. Jacobs, Stewart, Brown and Kimmelman measured the rate of hemolysis rather than the final equilibria through photographic methods obtaining a continuous hemolysis curve.[4] Another quantitative method involves the colorimetric comparisons of the hemoglobin from the hemolyzed red blood cells present in the supernatant fluid.[3]

This test involves a condition of both osmotic and diffusion equilibrium and is easily influenced by temperature and pH. As little as 0.5° C and 0.05 pH units change can have a measurable effect on the osmotic resistance of a red blood cell.[5] The oxygenation is also quite important as well as the relative proportions of cells and solutions, manner of mixing and the duration of exposure.[5] The degree of anemia can affect the test because, in a severe case, there are fewer cells available for destruction. The age of the blood and the CO_2 content are other factors to consider in the interpretation of the results. Chromagens in the blood, such as bile pigment, are hard to correct for in the visual determination of the beginning and end point of hemolysis. The anticoagulant used in the drawing of the blood is another important point which must be standardized in a laboratory method for the determination of the erythrocyte osmotic fragility. Many of these conditions can be controlled to enhance the reproducibility of the test.

In congenital hemolytic jaundice there is an inherent error in the visual determination of the beginning and end of hemolysis in a series of increasingly hypotonic solutions; the bilirubin in the blood plasma interferes with the color of the supernatant liquid. This is especially dangerous when one is comparing the patient's hemolysis to that of a control. It has been suggested that washing the cells of a jaundiced patient in saline and resuspending them in saline and treating the control cells similarly will accommodate

for this difficulty.[1] However, this is time consuming and it has been reported that washing and resuspending erythrocytes in saline changes their fragility somewhat, and, therefore, might invalidate the results of the test.[3] It is for this reason that the photoelectric test for osmotic fragility is a more accurate judge of hemolysis than a visual determination. In the use of the colorimeter, the patient's blood is not compared to a control. The grams of freed oxyhemoglobin in the supernatant fluid is read off of a graph for the colorimeter used. A blank is utilized which corrects for any color which may be present in the plasma.

The method under discussion is a modification by Hunter[6] of the technique of Waugh and Asherman.[7] It permits a quantitation of the osmotic fragility not possible in visual determinations of hemolysis. Because the amount of liberated oxyhemoglobin is proportional to the number of cells hemolyzed, the hemoglobin concentration can be used as a measure of hemolysis. It is inherently more accurate than visual determinations because it bypasses the difficulty encountered with jaundiced blood and is also more accurate than red cell counts for obvious reasons.

First of all, the pH, temperature, O_2 and CO_2 contents, and age of the blood must be standardized to make the results repeatable and the test more accurate. The hypotonic solutions are made with freshly distilled water and chemically pure sodium chloride. The sodium chloride is weighed on the analytical balance. The solutions are kept in glass stoppered bottles at room temperature and the pH is checked at two week intervals with the Beckman pH meter. The pH must be within a range of 5.5 to 7.0 and when it reaches an extreme end, new solutions should be prepared. They must be made up every four to six weeks and probably even more often in the summer to compensate for the greater evaporation. The saline concentration is checked by the silver nitrate method.

Two and a half to five cc of blood is collected without stasis, using heparin as an anticoagulant, and rotated gently to completely oxygenate.

Two one-hundredths of a cc of the whole unwashed blood is pipetted with a Sahli pipet into each solution and the pipet is rinsed with blood between each tube of the hypotonic saline (the blood used for the rinsing is discarded). If the pipetting is always done in one direction the error from contamination is standardized and the error between the solutions is too small to be significant. The blood should always be pipetted from the 0.85% concentration downward. Each tube is inverted several times and left at room temperature for one hour. The test tubes are then centrifuged at 1500 revolutions per minute for ten minutes and the supernatant fluid is then decanted into Evelyn colorimeter tubes and read with a 540 filter on the Evelyn colorimeter. The 0.80% supernatant is used as a blank as it should have no hemolysis. If there is suspected hemolysis in this concentration, a 0.85 or 0.9 percent solution may be substituted for use as a blank. This use of a blank will correct for any interference from bile pigments and/or other plasma constituents which might alter the light absorption. A tube containing distilled water and 0.02 cc blood is used as 100 percent hemolysis and denotes the total grams of oxyhemoglobin present. The colorimeter readings are then translated into grams of oxyhemoglobin. Quantitation is possible with this method if the results are given in percent of the total amount of red blood cells hemolyzed in each solution and if the increment between the adjacent hypotonic solutions is reported. If the percent hemolysis is plotted on the

ordinate against decreasing strength of saline along the abscissa, a sigmoid curve is obtained.

The percent hemolysis is obtained by dividing the total hemoglobin concentration (reading of the distilled water tube) by the hemoglobin released in a specific concentration of saline. (The hemoglobin concentrations are derived from the colorimeter readings.) The use of a hemolytic increment allows one to read the cells which were hemolyzed in a particular solution but not hemolyzed in the previous solutions. It is the "rate of progress of hemolysis from tube to tube".[3] It has been suggested that in certain pathological conditions irregularities in the hemolytic increment might represent changes in the "thickness population" of the blood.[3] The hemolytic increment is the increase in degree of hemolysis from one tube to the one next to it, the additional amount of hemolysis occurring in successive tubes which contain diminishing salt concentrations. For instance if tube number four had ten percent hemolysis and tube number five had fifty percent, the increment of tube number five would be forty (fifty minus ten). When the hemolytic increments are plotted, one can not only report that the osmotic fragility of a patient's blood is increased or decreased but can show a "picture" of the hemolytic process. The curve will show where the hemolysis begins, where it is complete, the mean corpuscular fragility (the concentration where fifty percent hemolysis occurs), the height of the highest hemolytic increment, and the breadth of the curve. In this type of graphic presentation, the hemolytic increment is plotted on the ordinate and the percentage of saline on the abscissa. The curve can be monophasic (practically all of the cells hemolyzed at one point, isohemolysis) or bi or multiphasic (hemolysis over a wide range, anisohemolysis). These are the characteristics which it is important to report for they are diagnostic. In normal blood the curve is monophasic and the highest increment is in a solution between 0.52 and 0.44 percent saline. The 50 percent hemolysis is usually in 0.50 or 0.48 percent.

In hemolytic anemia a "shift to the left" of the curve occurs. The hemolysis in relatively concentrated solutions is increased. The curve has a high peak and a narrow base.

In Mediterranean anemia there is a broad base and a shift to the right. There are no high peaks, but many small ones signifying a great diversity in the cell thickness.

The graphic reporting of the hemolytic increment can, therefore, distinguish between two diseases which have an increased fragility. It can distinguish between bloods showing spherocytosis and bloods containing target (thin) cells. It shows hemolytically different groups of cells, i.e., differences in red cell population thickness.[3] This reporting of the hemolytic increment is one of the more important values of a photoelectric determination of the osmotic fragility of erythrocytes.

In using this method of reporting one eliminates the need for correction for severe anemia, too, because the percent of the total amount of red blood cells hemolyzed is utilized.

The calculations are relatively simple. First the colorimeter readings are translated into grams of hemoglobin liberated in each solution. Next, the percentage of hemolysis is determined by dividing the total amount of oxyhemoglobin by the amount of hemoglobin released in the tube of saline in question. The increment is then calculated quite simply by subtracting the

percentage hemolysis of tube number one from tube number two, the second tube from the third and so on.

In conclusion, the benefits of a photoelectric determination of the osmotic fragility of erythrocytes are fourfold:

1. It allows for a quantitation of the fragility through the use of a graph depicting the hemolytic increments.
2. It automatically corrects for bile or any other chromatic substance present in the blood.
3. No correction is necessary for an acute anemia.
4. It presents characteristic graphs of different types of increased erythrocyte fragility.

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AN ELECTRONIC RELAY COLONY COUNTER FOR VIRULENT ORGANISMS*

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Several recording colony counters in use at this laboratory with avirulent organisms require touching a colony with a probe which activates a counter through a sensitive relay. This type of counter was not considered safe with virulent organisms for two reasons: (1) a small amount of current (approximately 4 milli-amperes) passed through the colony upon contact with the probe, frequently causing a small puff of steam which could conceivably disperse some live organisms into the atmosphere; (2) the voltage required to operate the relay through the resistance offered by the colony and agar plate was high (110 VDC). Although it is possible to isolate the probes from the line by a resistance, a potential exists at the probe which is sufficient to shock an individual mildly or to arc upon contact with ground.

An instrument has been designed in this laboratory which overcomes these potential hazards through the use of an electronic relay and an isolating transformer. The device requires an insignificant triggering current (less than 2 micro-amperes) and the probe leads are completely isolated from the supply line.

A schematic diagram of the instrument is shown in Figure 1 and a list of the required parts is shown below. The electronic portion (Figure 2) may be installed readily in an American Optical Company Quebec Colony Illuminator with the Veeder-Root counter surface mounted. Alternatively, all items may be mounted in a Bud Minibox (CD-2105-3 x 4 x 5). Cost of parts as listed will be about $27.00.

To operate the instrument, the bias is adjusted by the potentiometer (R_3) so that plate current, sufficient to close the sensitive relay, flows when the probe circuit is open. When the agar contact is in place at the rim of the petri dish, touching a colony biases the tube to cut-off and the plate relay activates the counter.

Parts List (See Fig. 1)

R_1 15 meg ½ watt
R_2 2.2 meg ½ watt
R_3 15 K potentiometer, Clarostat N32S.
C_1 20 mfd 150 VDC, Cornell-Dublier BR 2015A.
J_1, J_2—banana jacks
SW_1 SPST toggle switch
Relay, 10,000 ohm SPDT Potter-Brumfield LM 5.
Counter, Veeder-Root, T-120506 110 AC.
V_1 6C4
T_1 110 VAC pri.; 135, 0.015A; 6.3V, 0.9A. Triad R-2C.
Rect. Selenium rectifier 65 MA, Federal 1002.
Miscellaneous—2 banana plug-ended probe leads, one terminated with small alligator clip, other with needle point test prod.
16 ga. sheet metal for chassis
Radio hook-up wire.

* This work was supported through an inter-agency agreement between the Office of Naval Research and Camp Detrick, Maryland.

The opinions contained in this report are not to be construed as reflecting the views of the Navy Department or the Naval Service at large. (Article 1252, U. S. Navy Regulations, 1948).

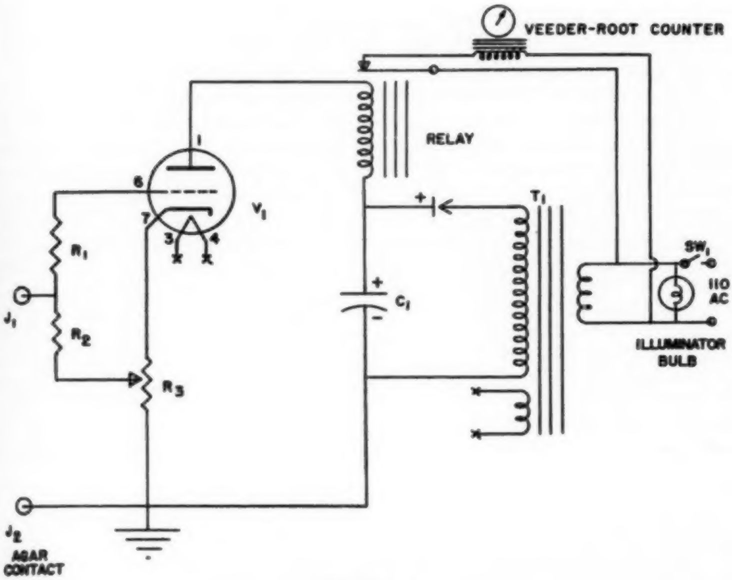

FIGURE 1
SCHEMATIC DIAGRAM

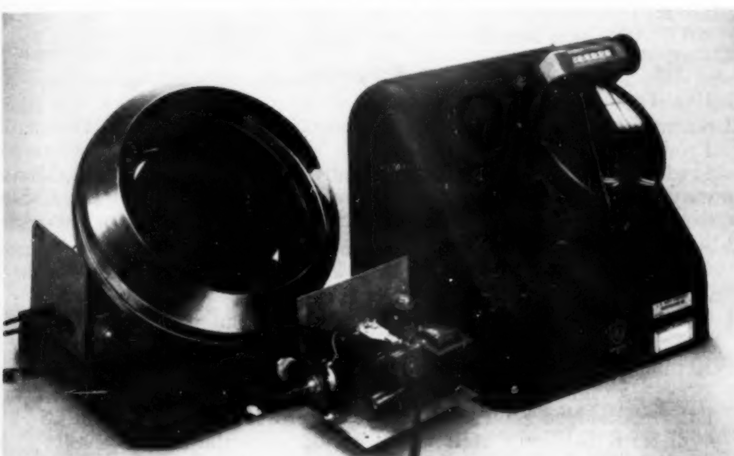

Figure 2—Disassembled view, showing parts placement of electronic components and their location in the base of the illuminator. The illuminator cover was notched to expose the two probe jacks and the switch.

A MICRO METHOD FOR BLOOD SUGAR USING ANTHRONE*

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The following method of blood sugar estimation was used in our laboratory for determining the glucose tolerance curves of parabiotic rats. Since micro technics are in demand for small animal work, whether in the research laboratory or in the pediatrics department of the hospital, we thought it worthwhile to publish our procedure for possible clinical use.

The method is based on the reaction of anthrone with carbohydrates to form a blue-green compound, first described as a qualitative test by Dreywood[1] in 1946. Quantitative tests for carbohydrate materials such as dextran,[2] insulin,[3] glycogen,[4] fructose[5] and glucose[6,7,8,9,10] have been devised by various investigators utilizing the basic reaction. Our procedure is based on Morris'[9] method, with the addition of an anti-oxidant, thiourea, to the reagent as suggested by Roe.[11] The basic procedure was modified by us for use with .05 ml. samples. According to a recent study by Handelsman and Sass,[12] the normal range of blood sugar values obtained with anthrone closely parallels values obtained with copper reduction methods.

Equipment

1. Pickard-Pierce micro pipets, calibrated "to contain" .05 and 0.10 ml.
2. Pyrex conical centrifuge tubes, 15 ml., etched at 5 cc. mark
3. Pyrex ignition tubes, 25 x 200 mm. (Note: size is critical).
4. Burette, automatic-zero type with bulb and reservoir, 25 ml., Pyrex.
5. Photo-electric colorimeter or spectrophotometer with 620 wave length setting, and cuvettes with inside diameter of 12-22 mm. (A Beckman model B spectrophotometer was used in this laboratory with matched shell vials, size 23 x 85 mm. serving as cuvettes).

Reagents

1. 10% Trichloracetic acid.
2. Anthrone Reagent. Dissolve 0.2 gm. anthrone (Eastman Distillation Products Industries, Rochester, N. Y.) and 1 gm. thiourea (reagent grade) in 100 ml. concentrated sulfuric acid of highest purity. Mixture may be heated cautiously to dissolve, but not above 80 degrees C. Store in brown bottle in the refrigerator. Solution may be used for two weeks.
3. Stock Standard Glucose Solution. Dissolve 0.5 gm. anhydrous glucose C.P., in 100 ml. saturated benzoic acid (0.25% solution). Keep in refrigerator. (1 ml. equals 5 mgm.)
4. Dilute Standard Glucose Solution. Dilute 1 ml. of Stock Standard to 100 ml. with distilled water. Make up daily. (1 ml. equals .05 mgm. or 50 micrograms).
5. Working Glucose Standards. Add 0.5 ml., 1.0 ml., 2.0 ml., and 2.5 ml. Dilute Standard Solution to Pyrex ignition tubes, and dilute each to 4 ml. with distilled water. These standards contain respectively, 25, 50, 75, 100, and 125 micrograms of glucose and correspond to values of 62.5 mgms.%, 125 mgms.%, 187.5 mgms.%, 250 mgms.% and 312.5 mgms. % in this procedure).

* This project was supported by National Science Foundation grant G2843.

Procedure

1. Collect duplicate .05 ml. blood samples from a finger puncture using Pickard-Pierce pipets, and rinse each 05 ml. sample into a 15ml. conical centrifuge tube, containing 1 ml. distilled water. (Avoid contamination of specimen with cotton fibers).
2. Add 2 ml. 10% trichloracetic acid to each tube and mix. Add distilled water to 5 ml. mark etched on tube and stir with glass rod.
3. Centrifuge at high speed for 5 minutes.
4. Using a 4 cc. volumetric pipette, carefully withdraw 4 ml. clear supernatent fluid and transfer to a 25 x 200 mm. Pyrex ignition tube.
5. Set up a blank consisting of 4 ml. distilled water, and working standards (as previously described) in Pyrex ignition tubes. (The number of standards set up is optional).
6. Slowly add 8 ml. anthrone reagent from a burette to blank, standards, and tests. The addition should be no faster than 45 seconds and no slower than 2 minutes with constant and vigorous agitation of the tube. (The use of a burette is strongly recommended, not only because of the added safety, but also because it facilitates control of the rate of flow of the solution. Most burettes will deliver 8 ml. of anthrone reagent within the time limits specified. If a burette is found to be satisfactory in this respect, its use eliminates the need for individual timing of tests, and assures uniformity of technique). Solution will look cloudy at first; then will clear to a light yellow-green which deepens to a blue-green on standing. Incomplete mixing or too rapid addition of the reagent will cause "charring" and tests will become a yellow-brown color. Such tests are worthless and must be discarded. Blank should be a clear yellow color.
7. Set tubes aside to cool in air, protected from dust, light, or gross contamination by aluminum foil covers until they reach room temperature.
8. Transfer solutions to cuvettes for reading in photo-electric colorimeter at 620 wave length. Adjust instrument to 0 optical density or 100% transmittance with blank, and read all tests and standards against this setting.
9. Make up a calibration curve on appropriate graph paper with readings of standards and obtain values for unknowns from curve if large numbers of tests are being run. (Curve is valid for immediate comparison only, since slight differences in concentration or purity of the sulfuric acid, and oxidative changes in the reagent after its preparation cause readings of standards to vary from day to day). For single determinations, comparison of the optical density of the unknown with the standard nearest it in color is sufficient, according to the formula:

$$\frac{\text{Density of the unknown}}{\text{Density of the standard}} \times \begin{array}{l}\text{Strength of standard used} \\ \text{for comparison in Mgms.\%}\end{array}$$

Note: Values above 350 mgms.% cannot be read accurately in the usual photo-electric colorimeter because of the deep color development at such concentrations. If high values are suspected, one filtrate may be run in the usual manner, and the duplicate run "on the half" i.e., on 2 ml. filtrate plus 2 ml. distilled water, and the answer multiplied by 2. An alternate procedure, as suggested by Zipf and Waldo,[10] is to dilute 4 ml. of the final solution with 4 ml. of a 1:1 mixture of sulfuric

acid and water, read, and multiply answer by 2. Running on half amounts is to be preferred over dilution of the final solution whenever possible.

Conclusion

A micro technic for blood sugar estimation using anthrone has been described. The method is a composite of several others, and scaled to an initial blood sample of .05 ml. It has been used in this laboratory for over 4,000 blood sugar determinations with a reproducibility of plus or minus 5%. Its advantages lie in its speed and simplicity. After deproteinization, only one reagent is required, a minimum of equipment is needed, and no heating is necessary.

The handling of concentrated acid may be considered a disadvantage of the method, but the use of a burette with a reservoir lessens this problem considerably. The sensitivity of anthrone, while desirable in a micro technic, increases the possibility of error due to contamination of tests by cork, cotton, paper, or similar materials. With reasonable care, and protection of samples by aluminum foil covers, the error due to such contamination should be minimal.

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USE OF PRESSURE TAPE LABELS IN THE BLOOD BANK: TO AVOID POSSIBLE TECHNICAL ERRORS AND HEALTH RISKS*

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Pressure tape has been successfully used for bottle labels and binding material at the University Hospital Blood Bank. The tape is a strip of paper impregnated with vinyl plastic and coated on one surface with an adhesive. With moderate pressure this sticks tenaciously on nearly all dry surfaces. It is not necessary to moisten the adhesive, thus casual contact of blood with the mouth and hands is avoided during processing

Figure 1—a. Dispenser arrangement for using rolls of pressure labels and tape.

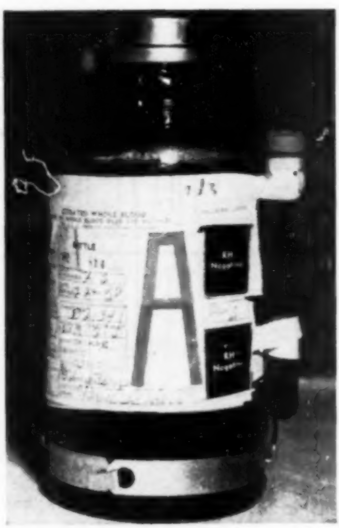

Figure 1—b. Labeled bottle ready for release.

and the possibility of transmitting infectious agents (e.g. hepatitic virus) to laboratory personnel is reduced.

The labels used by this laboratory are printed on rolls of tape with 250 to 1000 units per roll (Figure Ia). When they are removed from the roll they are placed on a firm flat surface such as a plate of glass. The necessary writing can then be easily done with a pencil or, preferably, with a ball point pen.

The main bottle label (Figure II) identifies the blood as to major blood groups. It is split into halves in order to lessen the necessary width of the individual tape roll and to provide a means to help avoid technical errors in processing and labeling the blood for recipients. The top half

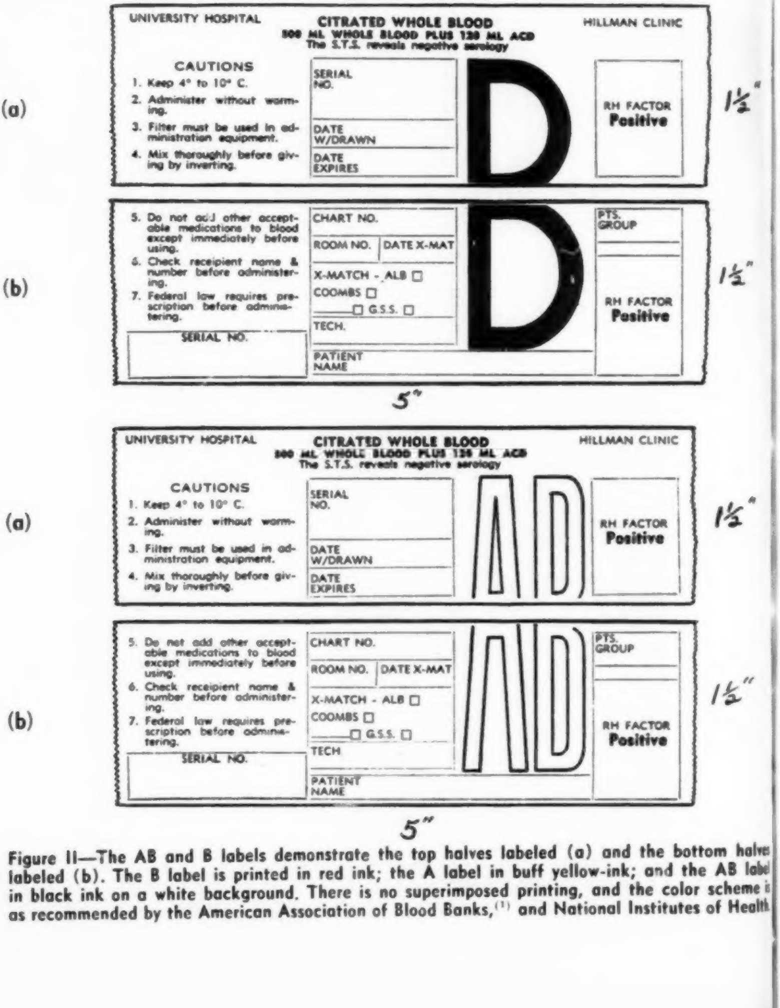

Figure II—The AB and B labels demonstrate the top halves labeled (a) and the bottom halves labeled (b). The B label is printed in red ink; the A label in buff yellow-ink; and the AB label in black ink on a white background. There is no superimposed printing, and the color scheme is as recommended by the American Association of Blood Banks,[1] and National Institutes of Health

has as its purpose the identification of donor blood. On it in bold print is the top half of the letter or letters indicating the major blood group. The other information is written in appropriate blanks before the label is placed on the bottle. The tape is applied over the original distributors paper label only after the donor sample has been properly processed. The bottom half has as its purpose the identification of the recipient's blood. It bears in bold print the lower half of the letter or letters representing the major blood group. It has blanks for proper recording of recipient and cross matching information as made available during the processing of the blood for a recipient. The completed bottom half is placed on the bottle under the top half and the major group lettering is matched to form one complete letter and bottle label. If the blood is not needed by the intended recipient, the bottom strip may be removed and the blood released for cross-matching with another patient.

Binding pressure tape (Figure IIIA) is used to fix external pilot tubes to each bottle before the blood is drawn. They are attached about one inch to the side of the original bottle label.

Tubes containing recipient blood samples are identified by labels placed lengthwise to facilitate writing (Figure IIIB).

Blood bottle serial numbering labels (Figure IIIC) are provided in groups or replicates of seven to fill the laboratory's particular needs for record keeping. This helps prevent errors in recording numbers. The serial numbering labels come in pads or rolls for easy dispensing.

The Rh negative label (Figure IIID) is used to clearly identify the Rh negative of both the donor blood and the cross matched recipient blood. It is placed over the Rh positive section on the top (donor) half and the bottom (recipient) half of the bottle label as each half is processed. Depending on the sera used and the results of Rh typing, D, C, E, d, c, or e, or an appropriate combination are written on the labels in the spaces above the Rh sections.

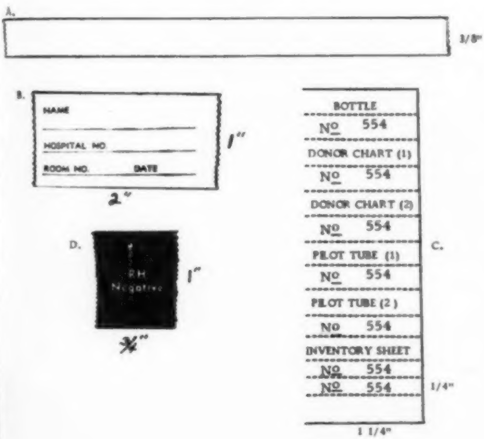

Figure III—
A. Binding tape for pilot tubes
B. Recipient blood sample tube label
C. Blood Bottle serial numbering labels
D. Rh negative label

The nominal cost of using the pressure taping and labeling operation as described above is approximately $0.05 per bottle. The complete major bottle label costs approximately $0.02 per bottle. This seems a meager and worthy expense for pressure tape and label* considering the total cost and charge for administering blood to patients and the convenience, protection, and technical efficiency and accuracy offered.

Summary

A method for using pressure sensitive type for blood banking labels at a nominal cost has been described.

Reference

(1) Technical Methods and Procedures of the American Association of Blood Banking: Minneapolis; Burgess Publishing Co., 1956, Page 63.

* The printed labels and binding material described in this paper have been supplied by the Bomar Printing Company, 923 2nd Avenue North, Birmingham, Alabama.

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ABSTRACTS

SEPTICEMIA OF THE NEWBORN DUE TO LISTERIA MONOCYTOGENESIS

Joseph Wiener, (U.S. Army Hospital, Fort Riley, Kansas) J. Pediatrics, 51, 392-403 (1957)

Actual diagnosis depends on the alertness of the bacteriologist. Cultures should be made from the blood (may be sterile even in septicemia), cerebrospinal fluid, meconium, urine, placenta, milk, lochia, pus, and exudates according to the case. At autopsy cultures should be made from all viscera, medulla oblongata. Agglutination titer fades rapidly after recovery —a rising or high titer (over 1:200) is significant.

The organisma as short gram positive rods usually in pairs or short chains sometimes may be mistaken for beta hemolytic streptococcus. It is easily decolorized and sometimes may be confused with Hemophilus influenzae. Its characteristics with differential media is outlined in sufficient detail. The identifying reactions are well within the resources of every bacteriology laboratory.

ATYPICAL ACID-ALCOHOL-FAST BACILLI CULTURED FROM HUMAN URINES

C. H. Lack, et. al., (Royal National Orthopaedic Hospital, London) Jour. Clin. Path. 10, 204-207 (1957).

An acid-alcohol-fast bacillus was recovered from the urines of 46 patients who have skeletal tuberculosis. The bacillus is non-pathogenic to guinea pigs but is capable of surviving in mice for at least seven weeks. It doesn't appear to conform to any of the saprophytes.

Morphological and cultural characteristics, biological, and drug sensitivity tests are presented in detail. In liquid Glover's blood media it grows in amorphous masses; on glyceroil-containing solid media it forms smooth, moist, buff-coloured colony and grows well at 60° C for four hours. Its lack of pigment and its inability to ferment rhamnose and arabinose and other features distinguish it from *M. smegmatis* and *M. phlei*. It differs from isoniazid-resistant tubercle bacilli in that it combines catalase production with non-pathogenicity for guinea pigs, but growing at 22° C on glycerol-containing media is the important characteristic that distinguishes it from drug-resistant variants of *M. tuberculosis*.

A QUICK METHOD OF PERFORMING THE PAUL-BUNNELL TEST

Frey R. Ellis, (Kingston Hospital, Kingston-on-Thames), Jour. Clin. Path. 10, 103-104, (1957).

The technique described gives reliable results 20 minutes after the test has been set up. When read at two hours the rapid test appears to be definitely more sensitive than the orthodox method.

The sera of 150 patients, suspected on clinical grounds of having infectious mononucleosis, were tested. The test was set up in duplicate and one set of tubes allowed to incubate and then stored overnight in the orthodox method of performing the test. The second set of tubes were placed on an automatic Kahn shaker and shaken for five minutes then allowed to stand for fifteen minutes. Readings are made at the end of that time and again in two hours.

When read at two hours the rapid test appears to show a higher titre in the late stage of infectious mononucleosis than the orthodox method.

"U" PROTEIN AND MIGRATION RATIOS IN PAPER ELECTROPHORESIS

Joel R. Stern, et. al., (Children's Memorial Hospital, Chicago), Clin. Chem. 3, 599-608 (1957).

This paper describes a protein component, migrating between alpha$_2$ and beta globulin which was originally observed in children with liver disease. Alpha$_3$ globulin would be the desirable name for this fraction but the authors point out that alpha$_3$ globulin has been applied to a more rapidly migrating fraction which has been found in viral hepatitis. The authors have designated this component "U" protein. The use of migration ratios in locating peaks of the alpha$_2$, "U" protein, and beta globulin, when these components resolve poorly, are discussed.

Characterization of "U" protein showed that it varies in its content of protein-carbohydrate complex and that it may be a lipoprotein. Evidence based on intential hemolysis indicated that "U" protein was not an artifact resulting from accidental hemolysis, but a protein which is found in serum under certain physiological conditions.

(Continued on Page 5

SPECIAL STAINS: THEIR NEEDS AND PROBLEMS*

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The use of histological stains other than haemotoxylin is generally known as special staining because such stains are used to demonstrate specifically bacteria, pigments, minerals, cellular secretion products, and enzymes.

These stains at times are of considerable help to the pathologist in determining the type of process going on in tissue and thereby aid in making a diagnosis. The results must be crisp in color reaction and free from precipitate. A control should always be run so as to leave no doubt as to the results. If the control fails to give a positive result, something is wrong with the stain or procedure. Many technologists fail to appreciate the value of special stains and sometimes may feel that they are only additional work and of questionable value. When the pathologist understands the use of these stains, a great deal can be accomplished by doing them. It is always a thrill when a problem in diagnosis is solved through a special stain a technologist has made.

Problems arise unless one is familiar with special staining techniques. It must be realized that if a result is poor, it is not always because the procedure has not been carried out properly. The stain may have originated in another part of the country or continent, and alkalinity and acidity of water may have some bearing on it. Not all companies put out the same grade of stain; this, as well as the different names under which dyes are produced must be considered. These are some of the reasons why stains must be modified depending upon the geographical locations and peculiarities of the laboratories in which they are done.

There are many methods published for special staining procedures. Some workers can produce beautiful results from one method, whereas another person will fail with the same method. One needs patience in doing special stains. If the results are poor the first time, the same method should be tried again. There are some stains that nearly all laboratories doing tissue work are called upon to perform. There are many questions raised when going over a procedure, and these are of concern to the beginner. (Insofar as each special stain may present its own problem, it is not the intention of this presentation to list in detail specific modification of staining techniques, but rather to give examples of the type of modification one may encounter. In this way it is hoped that anxieties concerning special stains may be overcome by the average technologist and encourage him to accept the challenge to delve into special staining techniques.)

Iron

This stain is usually requested when the haemotoxylin stained slides show the presence of a brown-colored pigment which may be melanin, formalin pigment, or iron. Laboratories using buffered formalin do not have the problem of the formalin deposit. In some cases both melanin and iron are present. To know just how much of the pigment is iron, an iron stain is done.

In some instances the identification of the pigment may have an important bearing on the diagnosis. There are various stains to show iron in tissue sections, but the Gomori's iron reaction[1] needs very little work, and through experience it has proven reliable. It has been discovered that there are points

* 1st SPF Award, Histology, 25th Anniversary Convention of ASMT, June 1957, Chicago, Illinois.

to remember when performing this stain. To an experienced technologist they may be quite obvious, yet when teaching students one realizes how important these points are.

1. Iron is slightly soluble in acid; it is desirable to use buffered formalin of pH 6.5-6.9.
2. While preparing the ferrocyanide solution, do not allow skin to come in contact with the stain.
3. Because of the reaction between metal and solution, all forceps must have their tips covered with paraffin. Any rust will cause a deposit that will react with the stain and leave a blue-colored artefact on the section.
4. Sections must be washed after the staining for at least five minutes so as to remove all acid. If this is not done the counterstain will not stay in the sections.
5. If the solution turns blue after addition of the hydrochloric acid, it may be due to a poor grade of hydrochloric acid which contains iron.
6. Rinse all glassware with distilled water before using to be certain it is (chemically) clean.
7. If it is found that the counterstain comes out when rinsed with water, blot sections and go straight into absolute alcohol followed by xylol.
8. Mount with permount, not Canada balsam, since balsam will fade the slides within a few weeks.

Methenamine Silver Stain

This stain was originally used for mucin and glycogen by Gomori.[2] Since then it has been found to be a splendid screening stain for all fungi.[3] Used in conjunction with a green counterstain, it gives a picture of sharply delineated, black staining structures on a background of green. Control of the stain may give results in which only the fungi stain.

Although this stain appears simple to perform, it takes time to experiment with it in order to achieve the desired results.

1. A control should always be run, and this should be checked at the half-hour period and again at the end of the hour. Again this may vary with the conditions in the laboratory, and trial and error method will give the optimum time to check sections for favorable reaction.
2. The temperature of the silver stain is quite important. It should be kept as close to 45-50° as possible.
3. After the solution is heated to this temperature it must be filtered, since a slight precipitate will form and collect on the slides.
4. If in some localities because of the water the stain needs more alkalizing, start with 3.0 cc. of tetraborate.

Fat

All laboratories at some time or another have performed a fat stain. There are various methods and dyes used, though by experimenting it has been found that oil red O[1] gives excellent results, especially for photography. A fat stain tells the pathologist how much of the lipid material is present and its distribution, both of which can be of great importance in some cases.

In teaching students, a change of methods was tried so as to make it easier to obtain good results.

1. Use a saturated solution of oil red O in solution of 50-70% alcohol.
2. Always filter the stain and keep covered at all times to avoid evaporation which will cause a precipitate to form.

3. When the fat stain is completed, float the section onto the slide, drain, and blot.
4. The counterstaining of the section can be done quite easily without the chance of destroying the tissue. Blot after the counterstaining and mount with Paragon mounting gelatin. This mounting media reduces the formation of air bubbles to a minimum.

Melanin

This pigment is a granular, dark brown pigment occuring normally in skin. In disease states it may occur in abnormal locations, even in lymph nodes. There are also tumors which produce melanin, and their identity may depend on the positive identification of the pigment. When a melanin stain is requested, it may be of great importance, and the technologist has to be familiar with the procedure. A section showing brown pigment has to be checked to see how much of the pigment is iron and what percentage, if any, is melanin. Melanin will bleach out when treated with potassium permanganate, but formalin pigment and iron will not.

A stain known as the Cooper stain gives good results and is an easy one for beginners. It is the rountine of many pathologists to request a melanin stain and a melanin bleach. It is the combination of results from both of these procedures that is necessary to identify the pigment, since not everything that gives a positive Cooper result is melanin. The section is first treated with 0.25% potassium permanganate for one to four hours and decolorized with 5% oxalic acid. The section is then stained with haemotoxylin and eosin. If the pigment is melanin it will be seen as black granules in the Cooper stain and will be bleached out with the use of potassium permanganate. In some laboratories the Fontana stain may be used instead of the Cooper stain, but in the hands of the inexperienced a precipitate may form, producing a poor result.

Reticulum

Generally the technologist despairs at the thought of doing this stain because very often the tissue will not adhere to the slides and because precipitate forms readily. This stain is used extensively in interpreting types of lymph node tumors, depending upon the reticulum pattern and the amount of reticulum present.

A method which has been modified to give remarkable results almost every time is Snook's reticulum stain.[4] In doing this procedure the most important point to remember is to always use plenty of distilled water for rinsing between each solution.

1. Use a silver stain that does not contain too much ammonium hydroxide, since this causes sections to become loose. The ammonium silver hydroxide solution used in the Gridley reticulum stain[1] has been found to be very satisfactory.
2. Rinse all glassware with distilled water before use (to be sure it is chemically clean.)
3. Using celloidin on sections sometimes causes poor staining of the reticulum.
4. Rinse three times before placing slides into the next solution. This guards against formation of precipitates as well as contamination of the solutions. The potassium permanganate and the silver ammonium hydroxide stain have to be made fresh each time.
5. If sections show a tendency to come off after being removed from the

silver, blot the sections, rinse carefully, and blot again after the sections are removed from the formalin.

6. Allowing the slides to stay in the absolute alcohol for fifteen minutes before taking down to water will also help to prevent the tissue from floating off.

7. If reticulum is toned too much, *do not* place the slides back into silver until they are *well* washed or a heavy precipitate will form.

8. If a Van Gieson counterstain is used, do not tone too much as the Van Gieson stain has the tendency to bleach the reticulum.

Mucicarmine

Mayer's mucicarmine stain[1] is considered excellent for the demonstration of mucin in sections. A haemotoxylin and eosin preparation may only suggest that mucin is present, and the special procedure becomes necessary to establish its presence. This stain may also play an important role in demonstrating mucin producing cells in body fluids, giving a clue to cancer cells having metastasized from some focus such as stomach or intestine. In this way the pathologist may suggest the possible site from which the tumor first arose.

There are problems in doing this stain, but if the following points are carried out, the results should be satisfactory.

1. After staining with Weigert's haemotoxylin there sometimes appears a dark discoloration around the slides. To clear this up, allow the sections to remain in the haemotoxylin for one minute. Decolorize a little with acid alcohol and blue. Allow sections to wash well in running water for five minutes; rinse in distilled water.

2. The metanil yellow counterstain will fade out if sections are allowed to stay in the rinse water longer than two minutes.

3. By expermimenting it has been found that if the sections are stained with mucicarmine for over an hour and then counterstained with the metanil yellow, the yellow color remains in the sections and gives a sharper contrast.

4. Blot the slides after taking them out of the mucicarmine stain. Rinse briefly in 95% alcohol to clear the slides. Do not allow them to stay in the alcohol long, for this will fade the red of the mucin.

5. If the mucicarmine staining solution develops a stringy consistency, discard it at once as it will given an all-over pink deposit resembling mucin, even after filtering.

6. If the mucicarmine stain tends to develop a stringy consistency, try storing it in the refrigerator. Experience has shown that this "stringy" consistency that may develop in the stock solution is due to molds or bacterial growth.

7. Instead of a dilution of 1:4, try 1:3. More striking results will be seen.

8. If the control does not show a good red color of the mucin, allow slides to remain in the stain; in some cases of skin, it may be necessary to leave sections of skin in the stain overnight.

The acid mucopolysaccharide stain[1] may supplement the results seen in the mucicarmine preparation because the staining contrast is striking. This stain should not be used alone but in conjunction with a mucicarmine. Although this stain is time consuming to make up, the procedure is quite

easily performed. The only point to consider is not to overstain with the picric-fuchsin stain.

Periodic Acid Schiff Stain

This stain is used for various reasons such as for demonstration of glycogen, polysaccharides, fungi, and alterations in blood vessels. When using it for fungi it must be understood that not all fungi have a affinity for this stain. It depends on the amount of polysaccharide material present.

This stain may cause some concern to technologists. A few variations have been made which prove quite satisfactory.

1. Blot slides dry before placing into leuco-fuchsin. This prevents the stain from turning pink and spoiling the reaction.
2. Always run a control. The leuco-fuchsin may be colorless but still may not work.
3. When using stain for fungi, counterstain with light green; this gives a more striking result.
4. Decreasing the time of the sulphurous acid rinses to one minute each and then wash for twenty or twenty-five minutes in tap water. This gives a brighter color.
5. Counterstain lightly with haemotoxylin. Allow slides to blue in cold water before dehydrating.

Trichrome Stain

Gomori's one-step trichrome stain[1] is considered very easy to perform and gives beautiful results with very little effort. Muscular tissue stains red with this stain, and this technique is generally used to see how much of a tissue is muscle and how much is fibrous tissue. When properly done, elastic tissue can also be seen.

1. If aniline blue is substituted for the green, the staining time must be cut to two minutes.
2. Placing slides in Bouin's solution at 56° for half an hour will make the colors more brilliant.
3. Be careful to always use acidified distilled water for rinsing *before* and *after* staining. This will keep the stain from fading.
4. The stain keeps well in the refrigerator. When muscle tissue begins to stain blue or green (according to which was used) discard the solution. (We have found one additional point which saves time and labor. By keeping mixed stain in the refrigerator the rate of deterioration is markedly reduced and stock may be kept for a longer period of time. As you know, stains kept on the shelf in a warm room soon evaporate and leave a thick residue.)

Elastic Tissue

Elastic tissue is very easily demonstrated with Verhoeff's stain[1]. The request for an elastic tissue stain is of importance. For example, when elastic stain is used on thyroid, it is done to show whether the blood vessels are being invaded by the tumor cells. Haemotoxylin staining will not show the elastic tissue of the smaller veins, which aids in identifying them.

1. An extended staining time does not matter. The longer you stain, the more decolorization is needed.
2. Decolorize the slide controlling the degree by using the microscope and discontinue when the background is the color of the 2% ferric chloride used in removing the excess stain.

3. If a Van Gieson counterstain is used, do not decolorize too much as Van Gieson will lighten the stain.
4. Elastic tissue stain will keep for many weeks if stored in the refrigerator. This keeps it from becoming thick.

Summary

Special stains are very often avoided by the inexperienced technologist. It is only by experience that perfection can be attained. Careful study of procedures, modification of them, and a great deal of patience may be necessary. Lurking around the corner of experimentation there is always the chance that new staining procedures may be developed by the conscientious technologist.

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A NEW SYSTEM OF LABORATORY RECORDING

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For the past six months the Firmin Desloge Hospital Clinical Laboratory Staff and hospital personnel have been trying out, and have found to be acceptable, what we believe to be a new method of laboratory recording. At present the method is being utilized in this 290 bed hospital, for the recording of approximately 240,000 clinical laboratory tests per year. The system has been devised to include the reporting of all hematological, microbiological, serological, biochemistry, urinalysis, and blood banking procedures, but does not include the reporting necessary for surgical pathology specimens. Two secretaries are necessary.

Before adopting this method, we had examined the methods and variations of recording now in use by most hospitals in the St. Louis area. In attempting to modify these methods for our use, we found in each some disadvantage involving inconvenience to our own personnel, the nursing staff who must maintain the records, the physicians who must interpret them, or the medical record librarian who must file and have them available for ready reference at all times. Among the disadvantages we found were the following:

1. Recording by a laboratory technologist on a patient's chart involves the use of a skilled employee for a non-skilled position, thus wasting valuable working time, and increasing personnel costs to the hospital.

2. Recording by laboratory secretarial personnel involves the transcribing of results by a person essentially unfamiliar with the tests. Erroneous recording is thereby amplified. In addition recording time is wasted since laboratory personnel must often spend time locating a record. The nursing and floor personnel are familiar with the location of the patient's record.

3. Recording on full-size printed forms increases the bulk of the charts, since constant repetition of a particular test, i.e., daily prothrombin activity or serial blood sugars, rapidly fills the allotted space on a sheet. Subsequent sheets become necessary, prior to complete utilization of recording space on pre-existing sheets.

4. Recording under the over-lapping requisition system in addition to increasing the chart bulk, requires additional time for removal of each report, if records are to be microfilmed.

It has been our purpose to institute a system which will, insofar as possible:

1. Minimize erroneous recordings of results
2. Maintain a legible, easily accessible report
3. Minimize the chart size
4. Facilitate micro-film photography
5. Reduce the time output of skilled technical personnel

The new system of laboratory recording which we have adopted is as follows:

The use of gummed adhesive strips, 1x7 inches, printed twelve to a page, and prepared for us by Avery Adhesive Label Company of Monrovia, California. You will note that this is indicated in Illustration I. Each strip has

a colored border as an aid in identifying the separate laboratory departments; i.e., red for Blood Bank, yellow for Hematology, blue for Chemistry, green for Bacteriology, and black for Miscellaneous. Before making a final decision in the adoption of this system, the medical record librarian consulted the Microfilm photo file organization in order that only colors suitable for microfilming were chosen. Each strip has a space for the name of the patient, record number, room number, recorder's initials, the date, and various tests. The tests are grouped on 22 different strips so that the frequently ordered combinations of tests appear on the same strip. For example, liver function tests as a group are all placed on one strip. Each strip has a perforated edge to facilitate separation from the stock sheet.

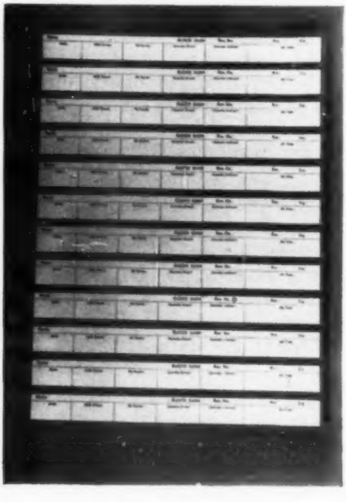

Illustration I

Sheet of recording strips as they are received from the company (factory). Note perforations between each strip.

Immediately on completion of a test or group of tests, the technologist records the results on the proper laboratory strip, fills in the other required information, including her initialed signature. Then the strip, together with the patient's requisition, is taken to the laboratory secretary who checks the report for completeness, and makes the laboratory charge which is sent to the business office. The strip reports are then placed in envelopes, and delivered to the respective divisions three times a day—11 A.M., 3 P.M. and 5 P.M.

The ward secretary on each floor separates the backing from the adhesive strips and attaches the strips to a specially ruled sheet. See Illustration II. On the back of this chart sheet are printed our normal laboratory values. Additional chart recording sheets are necessary only after

complete utilization of the pre-existing sheet space. See Illustration III. If the patient has been transferred to another division or discharged, the nursing service sends the strip to the proper floor, or the Medical Record Department. This same procedure of recording is followed in our Out Patient Clinic except that the medical record department assumes the responsibility for inserting the strips into the proper chart.

Illustration II
Method used in attaching strip to the base sheet. Note ruled base sheet with normal values on reverse side.

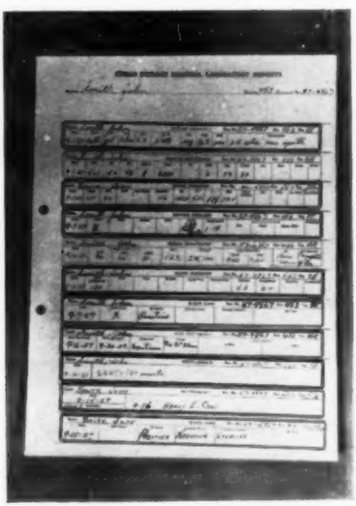

Illustration III
Laboratory report sheet as it appears in the patient's record.

We have found several distinct advantages to the strip recording system over other methods.

First There is minimal opportunity for recording an error in that the technologist who performs the work also makes the entry on the report form. While this does increase her total per unit test time in the laboratory, it eliminates her making repeated recordings on the patient's records at the chart desks on the various divisions. Our experience indicates that the technologists themselves prefer this method, not only because of the convenience but also because of the time saved.

Second Physicians prefer this recording system because the colored borders on the strips simplify the finding of specific reports.

Third This system utilizes space more advantageously than any method we examined. Charts are less bulky and may be stored in considerably less space.

Fourth All reports are readily visible for microfilming without undue manipulation or removal of any report from the record. The only significant disadvantage in the system involves the question of cost. To be sure there is an initial investment necessary for printing plates, however, if a group of hospitals can agree on the type of strip desired, the ordering of large amounts greatly reduces the ultimate cost per strip.

SUMMARY

We present this new system of laboratory recording which has proved to be more advantageous for us than other existing methods. A great deal of error in making entries on records has been eliminated, records are legible and easily accessible, skilled personnel's time is saved, and the records lend themselves with ease to microfilm photography.

ADVENTURE IN A SMALL CLINICAL LABORATORY*

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The purpose of this paper is to encourage other small clinical laboratories to set up training programs for student medical technologists. The advantages of such training programs, as we have found them, will be discussed in relation to

(1) The student medical technologist

(2) The hospital

(3) The laboratory personnel

(4) ASMT and ASCP

Our training program for student medical technologists began with a plan conceived by our pathologist. As a pathologist, he had been training medical technologists fourteen years. Student medical technologists, as he knew, are better than average educationally, more than average in physical attractiveness and of marriageable age. He had seen the statistics[1] borne out. The students become well trained, registered, work two or three years, become married and have families. Sixty-nine percent[1] of the girls would retire. Our pathologist felt that this retirement came just when they had become good medical technologists.

Our pathologist called two medical technologists who had recently retired to have families. One of these MT's was myself. He asked, "Would you like to come back to work?" and explained his plan. We could go back to work half a day, five days each week, our salary would be seventy-five percent of our former earnings. Our duties would include: (1) Instruction of student medical technologists in both theory and practice: (2) being responsible for the quality of work in the department in which we were teaching; (3) setting up new equipment as the laboratory grew; and (4) standardization of any new procedures the laboratory needed.

It didn't take much thought for us to decide to return to "active duty." As for me, the advantages were: (1) I had more free time with my babies, (2) I was doing work I enjoyed, and (3) the money was helpful.

The laboratory personnel liked the new program also because: (1) We were known to them; we understood the routine and would conform. (2) They no longer had the feeling they might be neglecting the routine and/or the students. And (3) they never seemed to have time to standardize new procedures or set up new equipment as promptly as they felt they should.

It was not easy setting up a sound and practical internship. We wanted to be careful never to allow the students to be used servicewise more than was necessary to teach them responsibility and give them confidence: however, we did not want to pamper them. We were approved for four students. We decided to take only one student in any three-month period. We felt that the students would benefit if each

* 1st Registry Award, 25th Anniversary Convention of ASMT, June 1957, Chicago, Illinois.

followed the same departmental schedule. We outlined the schedule of departments as shown in Table I, below:

TABLE I
School of Medical Technology
Departmental Outline of Courses

I.	Urinalysis, Gastrics, Pregnancy Tests	5 Weeks
II.	Hematology	8 Weeks
III.	Clinical Chemistry and B.M.R.	12 Weeks
IV.	Bacteriology and Serology	8 Weeks
V.	Bacteriology and Parasitology	8 Weeks
VI.	Blood Bank	4 Weeks
VII.	Histology	4 Weeks

We have found this particular departmental schedule most satisfactory. Each student begins with Urinalysis, Gastrics and Pregnancy Tests. Perhaps it would be better to call this time the orientation period. In this department, he studies not only the subjects listed, but is given time to learn the personnel, meet the doctors, acquaint himself with the hospital and physical being of the laboratory. One advantage of beginning in this department, too, is that, for the most part, these tests are qualitative and not dangerous. Inexperience that might be a hazard in other departments is not so pronounced here.

Hematology is the second department for every student. He is taught how to collect blood and the many other facets of Hematology. The fact that he learns to collect blood so soon, serves him well in subsequent departments. It is a simple matter to adapt himself to the proper collection of blood chemistries and blood cultures once he has overcome eight weeks of venipunctures.

We have, on occasion, varied a student's schedule after Hematology for the simple reason we do not want two students in the same department at the same time. No two students are the same—a slow one will hold a fast one back, unfortunately we have not had a quick one who could speed anyone up. Some students have great manual dexterity, others great capabilities in theory. Medical technology needs both. We insist that every student be proficient in the fundamentals but when we find one who is particularly adept in one department, we want to teach him all he can possibly comprehend.

You will notice here that we have our students in Bacteriology and Serology for eight weeks and Bacteriology and Parasitology for eight more weeks. This particular schedule gives us more time in Bacteriology and sufficient time in Serology and Parasitology. The student can work with one instructor in Bacteriology in the morning and the other instructor in Serology or Parasitology in the afternoon. The time element is not so critical in Serology and Parasitology.

I will mention here that after our students have completed Hematology, they are expected to take "first" call, with a graduate medical technologist on "second" call. This gives a student more experience and allows our "birds to try the wings." We want our students to learn early to accept responsibility. Medical technology is a responsible job and we must instill that in each student as quickly as possible.

Dr. Davidsohn's[2] suggested curriculum for Schools of Medical Tech-

nology proved itself invaluable. We adapted his suggested departmental outlines to meet our needs. (See Table II.)

TABLE II

Urinalysis, Gastrics and Pregnancy Tests

Student: ____________________

Date began: ____________ Date ended: ____________

Instructor: ____________________

Duration of course ________ weeks

Week	Material Covered	Weekly Grade
I	General Characteristics of Urine Qualitative Tests for Sugars Keytone Bodies Qualitative Tests for Protein Bence-Jones Protein, Proteoses and Mucin	
II	Quantitative Tests for Sugar Quantitative Tests for Protein Microscopic Examination of Urine	
III	Emphasis on Microscopic Examination of Urine Gastric Analysis	
IV	Addis Sediment Count Kidney Function Tests Other Chemical Tests: Bilirubin and Bile Acids Blood, occult Calcium Urobilin and Urobilinogen Detection of Urine Arsenic and Mercury Salicylates	
V	Pregnancy tests Friedman Frog Aschheim-Zondek	

Final Written Examination: ____________

Notebook Grade: ____________

Final Grade for Course: ____________

Remarks:

This shows our variation of Dr. Davidsohn's suggested outline in this department. For those who are not familiar with this publication, may I point out that we have removed the microscope and Parasitology and have substituted Gastric Analysis and Pregnancy Tests. We removed the microscope because we felt that the student would forget and not fully comprehend the subject at this time. Actually, every student

medical technologist has at some time used a microscope. We content ourselves with merely reviewing the rudiments in this department, saving the actual theory and "break down" until the next department, Hematology. As for Gastric Analysis and Pregnancy Tests, these specimens come to this counter in our laboratory and it is more convenient for us to teach them at this time.

We also adapted Dr. Davidsohn's required reading outlines. As a student enters each department, he is given a copy of this required reading. To assure ourselves that the student understands what he reads and to stress certain phases, we made lists of questions on each unit of each department. Each student is required to keep a notebook and include the answers to our questions. We review his answers and help the student correct any wrong or incomplete material. By having the student put this material in his own words, we can cover more ground faster and know at all times how effective our lectures are for the individual. An example of this is shown below.

TABLE III

CLINICAL CHEMISTRY
UNIT IV—SOLUTIONS

A solution is one in which the solute dissolves in a solvent to form a new substance. The solutes or solvents may be solids, liquids or gases, e.g.:

Solvent	+	Solute	→	Solution
Water	+	NH_3 (gas)	→	NH_4OH
Water	+	alcohol	→	alcoholic solution
Water	+	NaCl (solid)	→	saline

WATER IS CONSIDERED THE BEST SOLVENT BECAUSE OF ITS CHEAPNESS AND SOLUBILITY POWER. IN THE LABORATORY THE WATER IS USED CHIEFLY FOR THAT REASON. SOLUTIONS THAT HAVE WATER AS THEIR SOLVENTS ARE *AQUEOUS* SOLUTIONS, THOSE THAT HAVE ALCOHOL ARE *ALCOHOLIC* AND THOSE THAT HAVE ETHER ARE *ETHERAL* SOLUTIONS, ETC.

Define:

1. dilute solutions
2. concentrated solutions
3. saturated solutions
4. super saturated solutions
5. isotonic
6. hypotonic
7. hypertonic
8. collodial solutions
9. suspensions
10. standard solutions
11. working standard solutions
12. stock standard solutions
13. physiological salt solutions

PERCENT SOLUTIONS ARE SOLUTIONS THAT HAVE PARTS OF SOLUTE PER PARTS OF SOLVENT. E.G. TO MAKE 5% NACL SOLUTION, 5 GRAMS OF NaCl ARE ADDED TO WATER, ENOUGH TO MAKE 100 CC.

Problems—show all calculations

1. How would you make up 200 cc of 5% aqueous NaCl solution?
2. How would you make 100 cc of 7% alcoholic solution?
3. How would you make 100 cc of 15% sodium tungstate from 20% sodium tungstate?
4. If you had 150 cc of 20% trichloroacetic acid, and you wanted a 15% solution from it, how much water would you add?

The greatest problem which confronts any teacher is where to begin and how to progress. We realize that there must be some order and continuity. The way I begin to teach Clinical Chemistry to Student Medical Technologists is to review briefly the chemistry they have had. Most students are confident they remember very little and are awed or dread my department. They are pleased and surprised to realize that they do remember most of the essentials. It takes very little time, but gives the student confidence to consider the basic principles briefly. In addition, if there are weak points in the student's memory, this can be corrected before launching into a full scale course in the theory and methodology of Clinical Chemistry and perhaps completely losing the student. My experience has been if the student ever is "lost," he begins to dislike chemistry altogether and will do only what is necessary to get by.

We begin with reviewing the analytical balance, measuring devices, indicators and begin to review solutions. When the student feels sufficiently confident, usually in about three days or a week, he realizes he can use the analytical balance correctly. He knows the vocabulary and can make accurate solutions.

Cautiously I then begin to teach him of the proper means to collect and preserve blood for the different chemical constituents and before he knows what has happened, he is making a Folin-Wu filtrate. He is a clinical chemist!

Though more and more procedures and equipment are continually being added to our laboratories, we cannot afford additional time to teach them. (The shortage of medical technologists in our area is acute.) The student is ours for twelve months only. Realizing that it is impossible to cover all of the material necessary and have the student make a true evaluation, we made outlines of proper procedure in the more difficult departments, e.g.

Bacteriology

NOSE, ANTRUM, SINUS, EAR AND MASTOID CULTURES: Obtain two swabs. Make smear from one swab. Place second swab in broth and twirl rapidly to obtain material in suspension. Inoculate Brewer's thioglycollate medium and make blood agar streak-pour plates. If material is from ear or mouth, inoculate Littman's Oxgall Medium. Stain the broth culture at 24 hours. If gram negative bacilli are present,

inoculate either Endo's, EMB or desoxycholate agar, and a plain infusion agar plate. Original cultures may be made in Tryptose-phosphate broth or Brain-Heart Infusion Broth and Brewer's and blood plate made subsequently as indicated.

URINE CULTURES: Centrifuge specimen for 15 minutes. Pour off supernatant fluid and make smear and inoculate each of the following media with a loopful of the sediment: blood agar, Endo's or EMB, infusion broth (if patient is receiving sulfonamide therapy, use Brain-Heart Infusion with PAB).

STOOL CULTURES: Unless the fecal material is fluid, make a broth suspension of a small amount of stool. Inoculate a Bacto-SS Agar plate with 4-5 loopfuls of the suspension. Inoculate on Endo's or MacConkey Agar plate with 1 loopful of the stool suspension. To 10 cc of tetrathionate broth (with iodine added) add 1-3 grams of stool. Mix well. After 12-24 hours incubation at 37° C transfer 5 ml of the tetrathionate suspension to a petri dish (large). Add 20 ml of bismuth sulfite agar cooled to 45° C and observe after 24 hours for typical black colonies of typhoid. Pick a colony to nutrient broth. Plate for purity on Endo's Agar. Pick to broth then put on Kligler's and Citrate Agar. Plate again for purity, then inoculate differential sugar media. SS Agar plate should also be inoculated with 4-5 loopfuls of the tetrathionate suspension. Generally members of the dysentery group other than Flexner and Sonne are inhibited on bismuth sulfite agar.

SPINAL FLUID CULTURES: Spinal fluid from suspected meningitis constitutes an emergency. It is to be examined immediately. Do Gram stain first. If gram positive cocci are present, inoculate Brain-Heart infusion, blood agar plate and Loeffler's medium. If gram negative diplococci are present intracellularly or extracellularly, it is considered presumptive evidence of infection with Neisseria and is reported to the clinician immediately. Inoculate a chocolate agar plate and incubate in an atmosphere of 10% carbon dioxide.

The Bacteriology department illustrates again the problem of where to begin. We begin with the source of the material to be cultured. To us this seems the only logical beginning for the subject. Of course, later on, the gram positive cocci are separated from the gram negative bacilli, but in the meantime the student has learned the proper approach to the problems which one day will confront him when he is alone; he still has the causative organism when he reaches that particular "bug" in his study material. We feel that this is not only the fastest approach to this subject, but the most practical as well.

We often obtain our own material for culture. This is a particularly interesting and informative experience for the student. Too, it is another advantage of a small clinical laboratory.

Knowing that medical technologists need a general knowledge of some phase of medicine not strictly in our field, we gave them critiques, e.g.

NEOPLASMS: I. Definition: *A neoplasm or tumor is an autonomous new growth of tissue.* This simply means that a cell or a group of cells has broken through the normal restraints of growth and has become for all practical purposes independent as far as the general nutrition and metabolism of the patient are concerned.

II. Nomenclature: In general, tumors are named for the cell from which they arise by taking the root work of the cell and adding the suffix "oma." Thus a tumor arising from fibrocytes will be a *fibroma* and one arising from epithelial cells — an *epithelioma,* chondroyte — *condroma,* etc. All malignant tumors are called cancer. Those malignant tumors arising from epithelial tissue are called carcinoma, from squamous epithelium come epidermoid carcinoma and from glandular epithelium comes adenocarcinoma, etc. Those malignant tumors of connective tissue origin are called *Sarcomas.* From fibrous connective tissue comes fibrosarcoma; from muscle—myosarcoma, etc.

III. Etiology:

A. *Extrinsic factors*

1. Physical agents such as mechanical trauma. There are many reports of the appearance of neoplasms after a single or continued mechanical trauma, but in only a few is the cause-and-effect relationship established.
2. Ultraviolet rays: The higher incidence of carcinoma of the exposed parts of the skin in the white race and especially in the southern latitudes indicates a relation to sunlight, presumably the ultraviolet rays.
3. Infra-red rays or heat. Mice kept in a warm and humid atmosphere with a stable temperature develop fewer spontaneous tumors and the tumors grow more slowly. This is possibly correlated with the lower death rate by age groups from neoplasms in the southern U. S. than in the northern. Further proof of the relation of infra-red rays to the formation of neoplasms is given by the kangri (fire basket) burn cancer of Tibet. To help fight the bitter cold the natives wear a heated brick in a basket on their abdomen and epidermoid carcinomas of the skin appear in this region.
4. X-rays and Radium Rays. There remains no doubt that both of these, when given in adequate doses, will produce carcinoma of the skin in men. The latent period may vary from a few years to more than twenty years. There is first atrophy of the skin and then the tumor arises in the edge of the radiation burn.
5. Chemical agents. Coal tars have been incriminated as a cause of epidermoid carcinoma by Japanese investigators and skin tumor on the scrotum of chimney sweeps in England has long been known. Numerous other pure substances have been shown to be carcinogenic (carcinoma producing). Workers in analine dyes have a high incidence of bladder tumors. In a high percentage of people with carcinoma of lip and buccal cavity there is a history of the use of tobacco. In pipe smokers a line of separation of the effects of trauma of the pipe, the heat and the tars of the tobacco cannot be made. There is mounting evidence that carcinoma of the bronchus or bronchogenic carcinoma is more common in heavy smokers. A very high percentage of bronchogenic carcinoma is also found among the radium miners of Schneeberg, Germany. Thus again incriminating radium.

Please understand that we have no intention of trying to make pathologists out of our student medical technologists by giving them these briefs, but we do feel that they should have an understanding of the tissue sections they prepare and stain.

Such material as this makes a course more interesting—after all we want technologists—*anyone* can be a technician!

We use any and every good paper we can find which might help the student become a good medical technologist. Examples of these are Dr. Rudolph J. Muelling's paper *Flame Photometry,*[3] Dr. Opal Hepler's paper *Prothrombin Time*[4] and Dr. F. W. Sunderman's criticism[5] of methods used on specimens supplied by his laboratory service. We borrow films from our very cooperative drug houses and institutions, e.g. *Acid-Base Balance*[6] and *Schistosoma.*[7] When new equipment is set up, a student assists and profits by such experience.

We give weekly written examinations in each department. A comprehensive, consisting of one-hundred multiple choice questions is given on completion of each department. A final examination, also multiple choice type, is given when the student is ready for graduation from our School of Medical Technology. This is patterned after our Registry examination.

Our laboratory has many inconveniences, some of which have proven to be practical advantages, others we have tried to eliminate. Our problems may be the same as many other small clinical laboratories. Our solutions may be yours.

Our hospital is small, with a maximum capacity of two-hundred patients (50% surgical and 50% medical). We do not have medical students or residents and only four interns. We must draw all of our own blood. To us this is an advantage. Our students know our patients and see many of the patients' physical signs of disease. The test which the student later performs on the blood he, himself, has collected is personal, and the results are more meaningful to him.

We have a closed staff and no research laboratories. We must read of what other laboratories are doing in the newest methods, but we know our doctors, and they know us. They do not hesitate to stop by the lab. and discuss patients. They also tell us when they believe our results to be incorrect. While this is not gratifving at times, it is very helpful to the department and to the student. When our results might be in error, they are checked from every conceivable angle.

Our physical setup leaves much to be desired. The laboratory occupies one large room. There is no privacy. Noise in any part of the room echoes through every corner. This is irritating, but there is an advantage. It is possible for a student, or technologist, to know of any interesting or abnormal finding in any department. The hematological picture can be correlated with the findings in clinical chemistry. We can impress the significance of the accumulation of departmental reports on the same patient.

With a limited patient load, we lack quantity of positive specimens. We keep stock cultures in bacteriology, weigh out samples in chemistry or use one of the commercial preparations, such as Versatol.[8] Parasitology was a problem, however. We bought prepared slides and borrowed slides from the Communicable Disease Center.[9] We still needed positive speci-

mens. After all, there is nothing like finding your "own" parasites. We contacted the veterinarians in our vicinity. They were most cooperative. Now we can have amoebae, Necator, Ascaris, etc., in thirty minutes if we will go to the veterinary hospital.

Being an instructor requires much typing, grading, and making up of examinations. There is not time during the day, nor is there a typewriter available. Fortunately this can be done at home and at night.

Our greatest difficulty seemed to be recruitment and the "fatality rate" of our students. We had established a three-month probationary period for each student. During this time both med. tech. instructors would have worked with the student and our pathologist would have had several conferences with him. We three would meet and discuss the student. We would decide if we felt the student could do the work and if he could and would maintain the highest standards and qualities of Medical Technology. Of our first ten students, we lost five. In addition, we did not have the number of applications for our School of Medical Technology that we might have had. What was wrong?

We had always worked for recruitment of student medical technologists in general. We had gone to schools, shown the film, *Career, Medical Technology,*[10] sent out volumes of literature, participated in radio and TV recruitment programs and held our Laboratory Open Houses. What else could we do?

Our pathologist came to our rescue. He told us where we might find the answer to our questions. He suggested a meeting of interested pathologists, technologists, the dean of the medical school and college professors who dealt more closely with our prospective students. We contacted these people and invited them to lunch (at our pathologist's expense). Each member of the luncheon group told of his problems and made suggestions for solutions. The college professors were as interested as the pathologists and technologists. They helped us a great deal and it has been an advantage to know these people personally. Some of the points made were:

(1) Now there are only 50%[11] of the students entering the scientific fields that there were in 1950.

(2) Medical Technology courses, in the college curricula,[12] do not come until the student enters the Junior year. The student becomes discouraged and loses interest.

(3) Many prospective Medical Technology students drop out of the Medical Technology courses because they feel that they are asked to study much unnecessary and difficult material. They have no understanding of why they need these courses.

(4) It was suggested that summer work for these college students in medical laboratories might be helpful in keeping them in the field of medical technology. At the same time it would show them why physics, chemistry, mathematics and bacteriology are so necessary.

(5) Our efforts in recruitment were praised, but everyone agreed that we need more. Personal contact is most effective, it was generally believed, but the public's unawareness of our profession must be eliminated if we are ever to recruit the quality and quantity of medical technologists we must have.

(6) We would lose fewer students to industry if we paid them something during the internship.

Out of this conference, the untiring efforts of our pathologist and the understanding and generosity of our hospital administrator, we now have two very effective aids to recruit for our School of Medical Technology and reduce our "fatalities." We now have a scholarship of $1200.00 (to be dispensed at the rate of $100.00 per month) for our student medical technologists with outstanding undergraduate records and with degrees. Our hospital also agreed to subsidize two summer students each year.

We have tried the summer student plan and heartily endorse it. Your hospital may be interested. The summer student charts, files, makes media, runs errands, etc. He is paid the minimum wage (one dollar per hour) and works forty hours each week. During this summer the novice learns the duties and responsibilities of a medical technologist. If he dislikes the work, there is still time for him to change courses. (We have had only one student who realized his vocation was elsewhere.) He learns why it is necessary to take the required courses outlined for him in the college catalogs. He learns, too, the advantages of acquiring a degree.

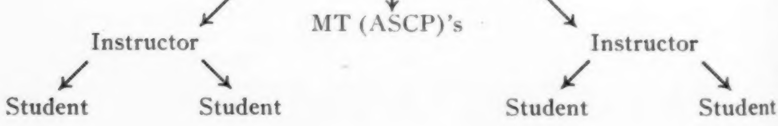
The advantage to the student medical technologist program is we get a good look at the prospective student's qualifications and do not have to rely on college records and a first impression to know if a student will become a good medical technologist.

The laboratory and hospital benefit in that many necessary tasks requiring no skill and much time, are done when those doing them routinely are on vacation.

To date we have had only one of these summer students return as a student medical technologist. She has been such an excellent student technologist and is now a registered MT (ASCP) and ASMT member of the highest caliber. We expect to repeat this performance in the future and we believe similar small clinical laboratories can do the same.

We feel that our entire student program is an overwhelming success and would like to see others profit by such experiences. The table below shows the

DIAGRAMATIC TEACHING PROGRAM IN OUR LABORATORY



Hospital Administrator: We are unusually fortunate in having a hospital administrator who is a former medical technologist. She has years of experience in hospital administration and though very busy, has time to come by our laboratory often to see our newer methods and equipment.

Pathologist: Here is the essential ingredient to any successful School of Medical Technology. He must be interested and respected by all. He must have the time and patience to listen to our problms and have the courage to chide us when we are wrong and defend us when we are right. He needs enthusiasm, not only for his own work, but enthusiasm which he can convey to those who work with him. We have such a pathologist.

Chief Medical Technologist: The chief medical technologist is an instructor extraordinaire. He is responsible for the over all lab. well being. He must be approached on any matter which might deal with the routine or special lab. precedent. He will assist any student when an instructor is not available.

Instructors: Medical technologists with several years' experience who work with the students. It is good if the instructors do not have the same "pet" department or specialty. In our case my instructor-partner has been a medical technologist fourteen years and especially likes Bacteriology. I have been an MT nine years and lean toward Clinical Chemistry.

MT's (ASCP): The backbone of the laboratory who do the actual routine and special tests but will still help "check out," e.g. "count behind" or check on identifications any time for a student.

Students: We ask for a college record, an acceptable evaluation by the Registry and a personal interview to consider prospective student medical technologists. Recently we have added to our application form that the student pledge not to marry for six months after entering internship.

SUMMARY: A training program for student medical technologists is an advantage in a small clinical laboratory:

(1) To to the student—approximately 80%[13] of the hospitals in the United States are 200 beds or less. (This does not include the doctors' offices and clinics.) The greater percentage[14] of students will later work in this type of small laboratory. In a small laboratory they can obtain the best program for this type work.

(2) To the hospitals—they can keep the ever growing laboratory in the fast growing hospitals staffed with medical technologists well trained to do small numbers of a great variety of tests.

(3) To the laboratory personnel—with instructors devoted to the student program, they are relieved of the responsibility, yet are kept aware and stimulated by such a program. The standardization of new procedures and setting up of new equipment is done for them.

(4) To ASMT and ASCP—keeps medical technologists working longer, even with expanding families. More experience means better quality work, the basic aim of these fine organizations. Recruitment done for your own School of Medical Technology is more personal and consequently more productive.

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THE LABORATORY DIAGNOSIS OF VIRUS DISEASES*

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This paper is written to interest Medical Technologists in the study of viruses, in the types and limitations of tests available for the diagnosis of virus diseases and in some of the well-standardized technics used in the aforementioned tests, especially tissue culture

Originally "virus" meant "a poison"[2]. Iwanowski over fifty years ago (1892) studying tobacco mosaic, a plant disease, was the first to suspect the presence of a submicroscopic infective agent.[2] Stanley in 1935 studying the same disease, succeeded in isolating protein particles which he proved could reproduce themselves when reinoculated into healthy tobacco plants and cause the mosaic of tobacco.[8] Cowdry, 1925, first saw the "elementary bodies" which are believed to be the initial stages of the life history of a virus.[1] To date, many theories concerning the origin and nature of viruses are on record such as that viruses are: a) degenerate bacteria; b) intracellular parasites that have lost every life function except the power to reproduce; c) large protein molecules, components of normal cells that have somehow or other escaped control, and actually may not be living.[4]

If we compare viruses with bacteria, otherwise the smallest organisms we know about, we find many dissimilarities. Viruses measure only 0.2 microns or less[1] and are invisible with the light microscope. They readily pass through porcelain filters and except for the lymphogranuloma venereum-psittacosis group, they are not susceptible to antibiotics[4]. Viruses cannot be cultured except in living cells. Just as with bacteria, however, many viruses have group affinities and respond as a group rather than as individuals. That is, a test for one is a test for all in the group and not for one alone.

Prior to the advent of modern technics, a physician first ruled out bacterial infection and then made his diagnosis of a virus disease solely by the patient's symptoms. At present, many tests are available to help confirm a diagnosis. All of them concerned in some way with the effect the virus has upon the metabolic activities of the cells in which it grows, or the products of its activity. There are three categories into which these tests fall. The virus can be isolated by passing it through animals, as swiss white mice; by inoculating chick-embryo incubator eggs or by inoculating tissue cultures, and then testing the virus culture against known antisera. Serological tests, using the patient's own serum, may be performed using known virus strains as antigens. Again, histological sections and smears can be scanned for inclusion-bodies and aggregations of elementary bodies or electron micrographs can be made from similar ultra-thin preparations.

All specimens usually received in a hospital laboratory are suitable for the diagnosis of virus. It is essential that the specimen be taken care of immediately by freezing it in its container at the very low temperatures of —20° C. to —70°C. These temperatures can be obtained by mixing alcohol and dry ice. If only the ordinary refrigeration temperatures of about 4°C. are available, a specimen may be adequately preserved by first combining it with a glycerine mixture consisting of 50% glycerine and 50% saline containing 10% horse serum. Lyophilization by which the specimen is first frozen and then rapidly dehydrated in a vacuum is the method of choice. The

* Read before First North American Conference of Medical Laboratory Technologists, 24 Annual Meeting of ASMT, Quebec, Canada, June 1956.

virus is dried without being killed. If a blood specimen is received, however, part of it must be poured into a separate container and not frozen otherwise hemolysis would prevent performing a complement-fixation test on it.[4, 11]

When a specimen is to be inoculated into animals, chick embryos or tissue cultures it must be in a fluid state, uncontaminated by bacteria or debris, and it must be concentrated. One authority recommends that, whatever the specimen, it be placed in its entirety in a deep-freeze box, frozen hard, and then while still frozen, broken up and ground to a smooth paste with sand. Infected chick embryo membranes or tissues taken from terminal cases of poliomyelitis and rabies are processed with a tissue grinder. These are washed in sterile saline and sometimes cut into fine pieces before putting them into the grinder.[7]

When a specimen is obviously contaminated, as pus for instance, it is weighed and 500 units of mixed penicillin and streptomycin are added to each ml. of raw specimen or 100 units of each antibiotic may be added separately to each ml. of prepared inoculum. Since members of the virus group lymphogranuloma venereum-psittacosis succumb to antibiotics, this method must not be used. An alternative method for killing the bacteria is the use of ether. The specimen is covered and allowed to stand for 18-24 hours. However, the ether treatment will kill the viruses of encephalitis and measles. It is necessary to be alert and proceed according to the nature of the organism suspected. If ether is used, it must be evaporated before beginning to prepare the inoculum.[4]

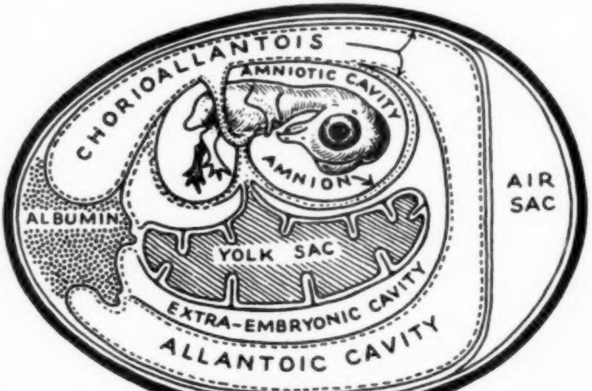
The specimen whether fluid or homogeneous paste, is now diluted to ten times its volume with one of the following: sterile water, buffered phosphate solution (pH 7.6) or a 10% beef-infusion broth in physiological saline. It is washed twice using the centrifuge for 30 minutes each time at 3500 r.p.m. The debris and bacteria are sedimented, the virus remains in the supernatant fluid.[2] This suspension is now known as the inoculum. To be certain that no bacteria are present, a sterility test is done by streaking it on a blood agar plate and incubating overnight at 37°C. If no growth appears by then, the inoculum may be used even though it may not be sterile.[2] While the inoculum is being stored overnight pending the results of the sterility test, 2-5% horse or rabbit serum or sterile milk is added to fortify it.[2]

In order to identify a virus by tests using antisera, it must first be isolated by growing it on living cells. There are three sources for these: tissue cultures; animals such as swiss white mice; and the embryo sacs of chick incubator eggs. Viruses have definite affinities for certain classes of tissues and are thus said to be "trophic." For this reason, it is important to consider the route of inoculation. For example, if it is suspected that the virus is a neurotrophic virus as rabies, intracerebral injections of swiss mice would be the method of choice. At least four young animals with soft skull bones should be inoculated at one time. After each mouse is anesthetized, it is placed on a table with its head pressed flat. Then 0.03 ml. of inoculum is injected at a point midway between the ear and the eye using a ¼" 26-gauge needle and a 0.25 ml. syringe. After a suitable time, the mice are killed and the selected tissues are ground using sterile precautions. A stock solution for later identification is made from the paste by diluting it ten times with salt solution. This is living virus, antigenic and infectious.

Incubated eggs containing chick embryos 4-14 days old provide an almost perfect medium for growing viruses. They are already sterile and can be kept

so with very little care. One needs to master the technics of inoculating the eggs and harvesting the membranes.[2,8] Beyond that, all that is necessary is an incubator to keep the embryos alive. To insure viability, the eggs are first "candled" or transilluminated. The position of the embryo and the air-sac are marked off on the shell with a lead pencil. There are several membranes within the chick-embryo egg: yolk-sac, amniotic sac, allantoic and chorioallantoic sacs.*[4] The probable identity of the virus and the manner in which the harvested membranes are to be used determine which of them is to receive the inoculum. Most commercial producers of virus antigen prefer to use the yolk sac. Wide-mouthed bottles make convenient holders for eggs during inoculation.[2,6,8]

Allantoic sac inoculations are simplest. The egg is up-ended with the broad surface on top. A short quick stab is made with a ¾" needle through the air sac. Yolk sac inoculations are made in the same way but the needle must be 1½" long and the embryonated eggs younger since younger eggs have larger yolk sacs.[2,4]



- Schematic diagram showing developing chick embryo and indicating cavities and other structures used for various routes of inoculation.

From The Review of Medical Microbiology, Fig. 88, p. 255 Lange Medical Publications[4]. Reprinted by permission of the publishers.

Two openings are necessary for inoculating the chorioallantoic sac. The egg is placed on its side and an area is marked off away from the embryo and the large blood vessels. The shell is carefully picked away from this area without puncturing the egg membrane beneath. A drop of saline is placed on the egg membrane and a slit is made in it using care not to puncture the underlying chorioallantoic membrane. The second opening is made in the air sac on the side of the egg and suction is applied with a small rubber

bulb. The original air sac disappears but at the same time the floor of the chorioallantoic sac drops and forms a new one. Now there is room for the inoculum which is put in with a sterile needle through the slit in the egg membrane.[4, 8] The needle should be held almost horizontally to avoid injuring the embryo. Ten day embryo eggs are used.

Amniotic sac inoculation is preferred for influenza. The chorioallantoic membrane is dropped as above, but the inoculum is put in with a 1½" needle. After the openings of the inoculated eggs have been sealed with hot paraffin or scotch tape, they are incubated at 36°-38°C. Progress of virus growth can be checked by removing samples from time to time with siliconed pipettes. Many viruses show characteristic plaques when growing on egg membranes. If cells growing in the embryo sacs are stained they may exhibit inclusion bodies. The harvested membranes are ground in a tissue grinder, diluted ten times as in preparing inoculum.[4, 7] The virus isolated from these extractions can be identified by complement-fixation or neutralization tests. Since viruses of both mumps and measles agglutinate red blood cells, the performance of a hemagglutination test is possible also.[10]

Tissue culture is the most efficient method for propagating virus for vaccines, or for identifying a virus in inoculum. Usually, the special tissue affinities of different viruses can be taken care of by growing them in cultures of cells from the proper sources. Sometimes the requirements are specific as will be seen. At other times, the trophic requirement is less important. Most known viruses pathogenic for man can be grown by any of the above methods.

Poliomyelitis grows best in tissue cultures. However, it requires a particular kind of tissue culture developed by Enders of Harvard (1948). By his method polio viruses can be grown in vitro on non-nerve tissue. The medium used to culture these cells consists of minced monkey kidneys embedded in plasma and spread over the walls of glass tubes or bottles and covered with a special nutritive growth fluid called Medium No. 199 (Morgan, Morton, Parker, 1950).[5, 13] In addition to balanced salt solution and other ingredients the medium contains a large number of amino acids.[11] The virus culture or suspected inoculum is placed in the tissue culture bottle. Soon the virus enters the proliferating cells feeding on them and causing them to disintegrate to release in 24 hours 300 virus particles per cell capable of reinfecting other cells. When the cells die they round up and appear refractile. This is known as the cytopathogenic effect. The use of this kind of tissue culture makes it necessary to replenish the kidney tissue by going back to the source again and again, requiring more and more kidney tissue from more and more monkeys. In the preparation of the Salk vaccine for poliomyelitis, the living virus is harvested and then killed with formaldehyde added in the proportions of 1:4. Three sterility tests are done on it: a) guinea pigs are injected for possible tuberculosis in the original monkey tissue used in the tissue culture; b) thioglycollate blood plates are cultured for the presence of bacteria;[13b] and c) both live monkeys and fresh cultures of monkey kidneys are inoculated to detect live virus.[5]

Other organ tissues can be used for culturing other viruses in similar fashion. However, all cultures where the cells are embedded in plasma with nutrient fluid placed over them present certain disadvantages. Sometimes the cell mass becomes detached from the vessel wall and floats free in the

culture fluid. Again, only the cells proliferating at the edges of the embedded masses can be seen, the rest are heaped up and opaque.[5] Many investigators thought that much more reliable information could be obtained about viruses if they could be cultured in cells arising from a single cell strain. Then the growth conditions could be controlled and the pattern predicted. In the search, Gey and associates at Johns Hopkins University discovered the remarkable "HeLa" cells.[4] "HeLa" comes from the first two letters of the first and last names of Helen Lane, patient of Dr. Gey, whose malignant cervical biopsy tissue furnished the cells. These cells are unusually uniform. When they are placed in a flat Pyrex glass bottle with nutrient fluid, they spread out in a single cell layer attaching themselves to the glass.[4,5] Moreover, after the tissue cultures have been infected with a virus, the progress of cell-destruction can be watched by placing the bottle on its side under the low power microscope without even opening the bottle.[5,11] Yet, the synthetic fluid can be withdrawn at any time to test it for neutralizing, hemagglutinating or complement-fixing antibodies. This also makes it possible to determine a change in pH below the optimum 7.3; an increase in the amount of residual glucose; or an increase in the ratio of floating (dead cells) to those still attached to the wall.[4,5] Any and all of these signs indicate that a virus is present. Once a good culture of HeLa cells has been established, the cells can be transferred to fresh bottles of nutrient fluid. This continuous processing of fresh cells requires the complete attention of one full-time technician who should be trained by an expert and not attempt to "pick it up" by himself. Pure cultures of virus can also be transferred from one bottle of HeLa cells to another as is done in making large quantities of vaccine. The HeLa cells are presently available only from research laboratories on application.[5]

All of the nutrient fluids used in tissue culture have as a base, a balanced salt solution (BSS). One of the best is Hanks' solution* which contains in addition to salts, glucose to furnish energy and an indicator.[3,11] The indicator, phenol red, is necessary because some of the viruses as poliomyelitis virus can live only within a narrow range of pH which it is important to maintain. The HeLa cells are very sensitive to metal contaminants found in the water of most laboratories, in some reagents, and in glassware. Accordingly, it is recommended that triple glass-distilled demineralized water (D.M.), sintered glassware and brand reagents be used wherever indicated since these have been empirically determined to possess the desired purity.[11] The complete growth medium contains in addition human serum, a mixture of antibiotics, a growth substance, and sodium bicarbonate. The source of human serum is people who are willing to sell their blood. Ascitic fluid withdrawn under sterile conditions may also be used, but not blood from a blood bank since it contains citrate.[5] The serum or ascitic fluid must be put through an ultrafilter of sintered glass and a sterility test done on it before incorporating it into the medium. Antibiotics are used to discourage bacterial contamination and two are more effective than one.[11] To promote growth, one authority uses chick embryo extract (EE).[9] and the authority represented by the method cited in the appendix uses yeast extract which might be more easily standardized and somewhat more convenient to use. During sterilization, the medium is likely to become acid. The bicarbonate is added to overcome any excess acidity.[3]

* Appendix.

It is general practise to replace the supernatant nutrient fluid on the cell cultures every 3-4 days. If the supernatant is not changed, maintenance solution consisting of 5% glucose and 1.4% $NaHCO_3$ must be added. After the tissue cultures have been growing 4-5 days, the cells may be harvested and distributed to tubes or flasks preliminary to inoculation with the virus. In order to loosen the cells from the sides of the bottles without injuring them, versenate solution* may be used. Hank's B.S.S. growth medium is decanted and a comparable amount of versenate solution is added. This is placed in a 37°C. incubator for 30 minutes, poured into a 50 ml. centrifuge tube and rotated at 1500 r.p.m. for 10 minutes. It is then poured off and replaced with a buffer solution.* The cells are resuspended by shaking thoroughly but gently. The HeLa cell harvest is then measured by counting in a hemocytometer the number of cells in 1 cml. The total volume of the supernatant is noted. All but one-fourth is poured off and replaced with enough buffer solution to make each ml. contain 600,000 cells. Five ml. amounts of the new suspension are distributed by means of siliconed pipettes into flat-sided bottles. If possible refrigeration temperatures are maintained to cut down cell loss. Then 100 ml. of growth medium is added to each bottle and the bottles are placed temporarily in a 37°C. incubator. From this stock suspension of cells new cultures can be replanted.[11] It takes about two hours for HeLa cells to become firmly attached to glass again.[3] Small bottles are used for cell culture, larger bottles when a known virus is being propagated as for a vaccine because large quantities are important. When testing for unknown viruses, small test tubes measuring 13x100 mm, Porter flasks, Carrell flasks and other smaller containers are used. The number of cells planted must be sufficient to establish a colony of contiguous cells and permit maximum growth potential.[3] No matter what the shape or size of the vessel, the ratio between the cells and the nutrient fluid and the ratio between this culture medium and the amount of virus injected must be carefully regulated in order to estimate correctly the end-points of activity of the virus in question. These end-points are measurable by a drop in pH, the amount of glucose not used by the culture cells after a period of time, and visible floating cells which are dead cells.[3]

The stock culture solutions are ready for inoculation with the virus. This, when it has been propagated, must be harvested and then titrated to find the measure of its growth as compared with the end-point of the original virus inoculum. The following methods by Scherer[9] for virus propagation and titration are reprinted together here in detail with permission of the publishers and they follow the method of propagating stock cell-cultures above:

A. Immediately dispense the cellular suspension with 1 ml. serologic pipette as follows (keep cells in suspension by mixing frequently):
   a) *For virus propagation*:
      0.5 ml to each of 2 13x100 mm. test tubes
      1.0 ml. to each of 2 Porter Flasks with 11x22 mm. coverglasses
   b) *For virus titration*:
      0.3 ml. to each of 15 13x100 mm. test tubes

Immediately slant cultures and incubate at 37°C. Three or four days later, add 0.3 ml. (6 drops) of human serum to each tube culture and each Porter

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* Appendix.

flask and incubate for 1-3 additional days before proceeding to step B.

B. *Infection of Cultures with Virus. Harvest of Virus.*

1. From Porter flasks and the 2 tube cultures for virus propagation, discard old medium. Add 1 ml. BSS. Tilt vessel to flow fluid over the walls. Remove liquid. Add BSS and wash twice more. To each Porter flask, add 18 drops and to each tube 9 drops of chicken S-10, MS-95.
2. Add 0.1 ml. virus (10% suspension of infected mouse brain or harvest from cultures of strain HeLa) to Porter flasks and tubes. Incubate at 37°C.
3. Place other tube cultures (titration series) at 30°C.
4. Daily for the next 4 days, examine microscopically the cells in virus infected cultures (tubes and flasks).
5. *When cellular destruction occurs:*
   a. Place at —20C. the 2 tube cultures containing virus.
   b. Remove and discard fluid from each Porter flask; add 1 ml. unsterile, warm, rinsing BSS. Place at room temperature for 20 minutes. Discard BSS with a straight pipette to a 13x100 mm test tube. Put Bouin's solution in each PF for 20 minutes. With bent tip platinum spatula, remove coverglass to Columbia Coplin jar containing 80% alcohol and proceed as per instructions for staining with methylene blue and fuchsia.* Examine for inclusion bodies.
   c. *Put tubes, Porter flasks, contaminated pipettes, etc., in discard tray (not in battery jars).*

C. *Measurement of Viral Growth*

1. *Preparation of Cultures for Titration*
   a) Label in pairs the cultures kept at 30°C: 10^1, $10^2 \ldots 10^6$ and control.
   b) Slant the 14 tube cultures in the edge of a rack and remove the old medium. Add 1 ml. BSS. Allow fluid to flow over walls of tubes. Remove liquid. Add BSS and wash twice more.
   c) To the 2 control cultures add 0.4 ml. CHS-5, MS-95. Stopper.
2. *Preparation of the Virus Dilutions*
   a) Add with a 1 ml. serologic pipette 0.9 ml of CHS-5, MS-95 to 6 separate 13x100 mm tubes. Label $10.^{1-6}$
   b) With the same pipette, add to the first tube (10^1) 0.1 ml. of virus. (See Section B, 5a.) Mix.
   c) With separate 1 ml. pipettes for each dilution, serially transfer 0.1 aliquots to make virus dilutions 10^1 through 10^6 leaving the pipettes in each tube.
   d) Add 0.4 ml. of each viral dilution to the tube cultures bearing corresponding numbers.
   e) Stopper the tubes, slant and incubate at 37°C.
   f) *Discard contaminated materials to trays.*
   g) One, three and five days later, examine the cells microscopically. The end-point of the titration can be taken as the highest dilution of virus that produces definite destruction of cells. To determine the amount of virus growth, compare this end-point with the titration end-point of the original virus inoculum reduced tenfold by the dilution that accompanied transfer of the virus inoculum to the culture tubes.* (Section B2.)

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* Appendix.

All viruses isolated by any of the above culture methods whether by animal, chick embryo or cells must be identified by a hemagglutination, neutralization or complement-fixation test. All require a specific antiserum to be used against the isolated virus antigen. Titration of the virus is equivalent to titration of the antigen. Antisera are usually available in research laboratories but a few are sold commercially.

The alternative and preferred method of identifying pathogenic viruses is to test the patient's blood serum for antibodies against a known antigen. General and hospital laboratories should not attempt to culture viruses and make their own antigens. Non-infectious antigens are commercially available for most of the usual virus diseases. The hemagglutination test now becomes the hemagglutination-inhibiting test. The neutralization and complement-fixation tests remain the same except that the antigenic source is outside the patient. A positive diagnosis with these serological tests is based essentially on the demonstration of a four-fold rise in the antibody titer of a patient's serum from the time of onset of the disease until 4-5 days later or as soon as antibodies have had a chance to develop.[10] Accordingly, as soon as the physician presumes his patient has a virus infection, a specimen of blood is taken and after 4-5 days another is taken and both are tested in the laboratory at the same time. Care must be taken that the blood is not hemolyzed. By now, the clinical symptoms are more firmly established. For best results, it is the responsibility of the physician to indicate to the laboratory which of the virus diseases he wishes investigated.[10, 11]

The complement-fixation test furnishes the most information in the shortest time. The test is performed according to the method of Casal which uses 1:2, 1:4 . . . 1:16 dilutions.[3, 4] Non-infectious commercial antigens include with their products details of the procedure which is essentially the Wasserman Test as modified by Casal.[4] The principle of the complement-fixation test is that if the patient has the disease, the antigen (virus) combines with the antibody in the presence of complement to bind it so that when indicator sheep cells are added with hemolysin no free complement remains in the patient's serum to hemolyze them. No hemolysis is a positive complement-fixation test. The degree of hemolysis is a measure of the amount of developed antibodies or titer. A four-fold increase in titer is significant for diagnosis.[4] Some viruses develop more than one complement-fixing antigen. In mumps there are two, both of which are present in sufficient amounts to give the required titer increase by the end of the second day.[4] Mumps without parotitis gives symptoms that resemble those of many other diseases and should be ruled out by doing a routine test for mumps in diagnosing all virus diseases.[10, 11]

A positive complement-fixation test, for instance, might indicate mumps meningitis but, at the same time, it would exclude the possibility of poliomyelitis since antibodies for the latter disease do not appear until the 18th day or later.[10] Complement-fixation tests will not differentiate between members of the lymphogranuloma venereum-psitticosis group because they are serologically closely related but antigens from any one of them can be used to detect the presence in serum of the antibodies of any other one.[4]

The hemagglutination test is specific for mumps and influenza, but it has fallen into comparative disuse in laboratories where complement-fixation tests for other virus diseases are being done also. The principle underlying this test is that while many viruses will agglutinate red blood cells, antibodies present in the serum of a well person or in one recovering from an attack

will prevent agglutination. The commercial antigen is titrated before testing patient's serum with a suspension of Type O red blood cells. The titer is the highest dilution of serum which will effect complete *inhibition* of hemolysis. This test requires two specimens taken at the begining of the disease and four to five days later. A positive diagnosis requires a titer rise of more than four-fold.[4, 6]

The neutralization test requires testing with live virus and is therefore beyond the scope of general and hospital laboratories because of the danger of infecting personnel. It is not a hard test to perform but it is expensive and time-consuming. The principle underlying it is that a given antigen will combine with its homologous antibody in equivalent amounts to neutralize its effect and if there is an excess of either component it can be measured. It is therefore, both qualitative and quantitative. Whether the technician has an unknown virus or an unknown antibody, the identity of the virus can be discovered when one places measured amounts of one with measured amounts of the other.[5] If one of the components is kept constant and serial dilutions are made of the other and these dilutions are inoculated into susceptible hosts such as young suckling mice, chick embryos or tissue cultures, the extent of the neutralization can be measured.[4] Suppose it is necessary to distinguish between the three types of Polio-virus:—I, II, and III. The patient's serum is tested against all three antigen types by inoculating tissue cultures:

1. Cells plus unknown antibody (patient's serum) . . . No change
2. Cells plus Polio I (antigen) . . . Cell destruction.
3. Cells plus unknown antibody plus Polio I (antigen) . . . No change because the neutralizing antibody is present in the same amount as the antigen. Since the patient has demonstrated antibodies for Polio I, Polio I is the infective agent.[5]

When mice are used for poliomyelitis testing, they are injected intracerebrally and a neutralization test is performed. The titer is based on the number of mice inoculated in each dilution which survive and the number of days of their survival. With some viruses, young animals die off in a few hours. The titer is calculated mathematically.[4] A titer increase of 100 or more over the initial titer at the time of inoculation with virus infected material is considered pathologically diagnostic. The neutralization index is the antilog of the difference between the negative logs of the control titer and the titer of the mixture of virus and serum.[4]

Infectious mononucleosis is now considered to be a virus disease although this has not yet been proven. A positive diagnosis of this disease depends on a positive heterophile-antibody test showing an increase in titer followed by an adsorption test with guinea-pig kidney (which must be negative), and another with bovine red cells (which must be positive).[4, 6]

In addition to the serological tests and isolation tests above, skin hypersensitivity test antigen and control sera are commercially available for mumps, lymphogranuloma venereum,** herpes, variola, vaccinia and serum-and infectious hepatitis. Directions come with the package. Skin test antigen, 0.1 ml. is injected intradermally into the right forearm and 0.1 ml. of control into the left forearm in the same manner. An erythrematous wheal develops between 24-48 hours. A positive test depends upon the extent of the wheal from the site of the injection and this varies with the virus.[4]

* Appendix.
** A Frei test is usually done in testing for lymphogranuloma venereum to supplement a positive complement-fixation test.

The third means by which viruses may be diagnosed is by finding inclusion bodies in the cells of smears or tissue sections, or from the coverslips placed in the flasks at the time the cell cultures were inoculated with virus.[11] Only a few inclusion bodies are by themselves distinctive enough to be conclusive for diagnosis although many of the known pathogenic viruses produce them. Some inclusion bodies are diagnostic only when they occur in specific cells. It is not clear what their significance is. They may represent a stage in virus life history preliminary to the dispersal of the elementary bodies; a reaction of the host cell to the presence of virus; or a substance manufactured by the virus as part of its metabolic activity in the host cell. The meaning may be different for each virus.

Elementary bodies are sometimes found outside cells as a result of cell disintegration. The electron or ultramicroscope must be used to see them since the largest is 0.2 mμ. This is too small to be resolved by the light microscope. Inclusion bodies are always within cells.[1] They measure 1-20 mμ and can be seen readily under oil immersion of the light microscope. Cowdry mentions "elementary bodies" as the smallest particles of virus that can be seen with the ultramicroscope. Molluscum contagiosum presents aggregations of elementary bodies that are easily visible under high dry power. Frequently their shape is spherical, but they may be square or brick-shaped. They may occur within the cytoplasm, within the nucleus or both—especially in the mononucleate cells. Since intracytoplasmic inclusion bodies are always embedded in a carbohydrate matrix, they may be differentiated from other material with the cytoplasm if they stain with Lugol's solution.[6]

Inclusion bodies almost always take the acid stain and are pink with hematoxylineosin stain wherever found. The classical example of an inclusion body is the Negri body diagnostic for rabies and found in the nerve cells of the horns of Ammon of the brain. These cytoplasmic inclusions are bright pink with thinner blue staining areas in them and appear more or less refractive. Inclusion bodies of other viruses found in the same location, as cat-scratch fever, are never refractive, have less definite boundaries, no internal structure of blue dots and stain pale pink.[7] Seller's stain is classical for the rabies inclusion body. The herpes type inclusion body is an example of an inclusion body that occurs in the nucleus. It stains pink, the nucleus stains blue. It appears to consume the nucleus gradually until only a nuclear membrane, characteristically ragged on the edges, remains. Still another type of inclusion body is that of molluscum contagiosum which is considered to be an aggregation of "elementary" or "initial" bodies in which these virus particles develop.[1,4] The only means at present, of diagnosing certain viruses as trachoma and inclusion blenorrhea, etc., is by means of inclusion bodies.[1,4] These bodies are similar to those of lymphogranuloma venereum and consist of eosinophilic particles that occur in round groups in the nucleus of an infected cell.[8] Inclusion bodies are sometimes useful for differential diagnosis. For instance, chicken-pox and small-pox may give identical results with serological tests, but the Guanieri bodies characteristic of smallpox are both intracytoplasmic and intranuclear, whereas those of chicken-pox are confined to the cytoplasm.[4]

When a patient has a virus infection in which inclusion bodies usually can be demonstrated but which diligent search does not reveal, they may be elicited quantitatively by injecting animals with the infectious material.

SUMMARY

Many of the tests and technics employed in the field of virology have been discussed, together with their limitations and the possibilities for their use in hospital and general laboratories. Tissue cultures provide many uses for virus study and other fields as well.*

All tests which do not involve the use of live antigens are suitable for hospital and general laboratories. A minimum of risk is encountered with the complement-fixation test where it can be used. Commercial antigens are available from many drug houses. The results are obtained more quickly than with the neutralization test and are considered as conclusive. Whenever no known commercial antigen is available the neutralization test must be done... usually by special research laboratories. Direct smears and sections of biopsy tissue are reasonably safe also. Otherwise, too little is known about virus pathogens to expose workers to the risk of handling them in living cultures.

Few hospitals engage consciously in any virus diagnosis as such at present. Since virus diseases are becoming increasingly better recognized and more important, it would appear that more advanced hospitals will soon make virus diagnosis part of their routine testing programs.

APPENDIX

1. Media for growing the HeLa cells.[11]
   (From the Division of Infectious Diseases, The Public Health Research Division of the City of New York)
   Caution: Use sterile precautions
   Run sterility tests on everything
   Use only demineralized (DM) water

A. *Complete Medium for Growth of Cells (stock)*
1. Hank's solution.......58%
2. Human serum........48%*
3. Yeast extract......... 2% (This is 5 ml. of liquid (Difco) yeast made up to 100 ml. with Hanks' solution.)
4. Penicillin and streptomycin... 100 units/ml. of medium. Add 0.29 ml. of mixed antibiotics to each ml. of medium.
5. Sodium bicarbonate (Abbott's)... 7.5% solution. Add 0.24 ml. to each ml. of Hank's solution.

* The serum must have been put through an ultrafilter of a sintered glass. Hanks[8] recommends that both in A above and C below, the sodium bicarbonate be autoclaved separately in a small vial inside the larger container which holds the other components and added after cooling.

A1. *Hanks' Balanced Salt Solution (BSS) 10 X stock*
1. $CaCl_2$............... 1.4 gms. dissolved in 200 ml. DM water
2. Glucose .... 10.0 gms.
   *NaCl .... 80.0 gms.
   KCl .... 4.0 gms.
   $MgSO_4 \cdot 7H_2O$ .... 2.0 gms.
   **KH_2PH_4 .... 0.6 gms.
   ***$Na_2HPO_4 \cdot 12\ H_2O$ .... 0.6 gms.
   Dissolve all in 2. in 800 ml. of DM water.

****3. Phenol Red, 2% ......100.0 ml.

* Use Merck's biological NaCl to avoid silver.
** May be contaminated with aluminum.
*** If the $Na_2\ HPO_4$ contains 12 H_2O, use 1.2 grams. Use only clear crystals and filter through sintered glass.
**** Phenol Red (0.2%): Dissolve 0.4 grams of dye in 22 ml. of N/20 NaOH. Bring the final volume to 200 ml. with glass-distilled DM water. Adjust the pH to 7.

Combine 1, 2 and 3 and bring to a final volume of 1100 ml., add 3-4 ml. of chloroform. Make 1x the solution and put up in 100 ml. quantities. Heat to drive off the chloroform. Autoclave at 10 pounds pressure for 10 minutes.

Preparation of Human Serum:

Freshly drawn blood is placed in a screw-cap flat-sided bottle that is lying on its side. When the blood has coagulated, the bottle is righted and allowed to stand in the refrigerator overnight. The clear serum is transferred to sterile test tubes, tightly stoppered and put back into the —4° C refrigerator. Slight hemolysis does not spoil serum for tissue culture.[4,5,11]

B. *Solution for freeing cells from glass of culture bottles*

NaCl	8.0 gms.
KCl	0.2 gms.
$Na_2HPO_4 \cdot 7H_2O$	0.6 gms.
KH_2PO_4	0.05 gms.
Disodium versenate	0.22 gms. (Obtainable from the Bosworth Chemical Co., Framingham, Mass.)

Add DM water to make 1000 ml. Autoclave at 15 (14.9 better if possible) pounds pressure for 15 minutes. Store in refrigerator until ready for use.[11]

C. *Buffer Solution (10 X stock to make 1000 ml.)*

Solution A: Dissolve the following in 900 ml. of DM water, add 3-4 ml. of chloroform:

NaCl	80 gms.
KCl	4 gms.
$Na_2HPO_4 \cdot 7H_2O$	2.0 gms.
KH_2PO_2	0.2 gms.
$MgSO_4 \cdot 7H_2O$	1.0 gms.

Solution B:

$CaCl_2$	1.4 gms.

Dissolve in 100 ml. H_2O (DM) and add 3-4 ml. of chloroform. Solutions A and B must be autoclaved separately at 15 pounds pressure for 15 minutes. To use add slowly with mixing, 10 ml. of Solution B to 90 ml. of Solution A.

Cleaning bottles:[11] Bottles may be cleaned by boiling them in 0.001% Dupanol, rinsing about 7 times in tap water then in regular distilled water and finally in demineralized water.

Sterility Test: One ml. of serum is added to a tube of blood thioglycollate[14] medium, kept at room temperature and read after 3-7 days.

Preparation of $NaHCO_3$ Used for neutralizing acid solutions and correcting pH in cell cultures.

$NaHCO_3$	3-5 gms.
Phenol Red (2%)	250 ml.
Water	22.5 ml. (Gives a 10% excess of water)

Dissolve and sterilize in 2-4 ml. amounts. The Na_2CO_3 can be reconverted to $NaHCO_3$ by bubbling CO_2 gas through it. The solution should be kept tightly stoppered.[3]

Antigens for the complement-fixation test may be purchased from Lederle Laboratories, Pearl River, N. Y., Microbiological Associates, Washington, D. C., and E. R. Squibb & Sons, New York, N. Y.

Seller's Stain for Rabies (U. S. Army Medical Manual)[12]

1. Make smears of gray matter of the brain on a slide or fix a small piece of brain (Ammon's horn) with cut surface up, on end of a cork stopper and make touch preparations by gently touching 3-4 times with a clean slide.
2. Fix for two minutes with methyl alcohol.
3. Seller's Stain:

   A. *Reagents:*

   1) Methylene blue .... 15 gms.
      Methyl alcohol ....100 ml.
   2) Basic fuchsin .... 32 gms.
      Methyl alcohol ....100 ml.

   Just before use, mix three parts of solution (1) with one part of solution (2) and five parts of methyl alcohol.

   B. *Staining technic:*

   "Touch" preparations are flooded with mixed stain for approximately 10 seconds, washed in water, dried and examined. The methyl alcohol serves as a fixative and no additional fixation is necessary. The chromatin should stain blue and the cytoplasm red. If a clear-cut differentiation of cytoplasm and chromatin is not obtained, additional amounts of either 1 or 2 are added until the desired effect is obtained. The stock solution should be stored in the refrigerator.

Machiavello's Stain for Inclusion Bodies[8]

Solutions: A. 0.25% basic fuchsin in distilled water
B. 0.25-0.5% citric acid freshly prepared
C. 1% Methylene blue in distilled water

Smears are fixed lightly in heat, stained for 3-5 minutes with freshly filtered fuchsin. The fuchsin is poured off the slide and then dipped quickly into the freshly prepared citric acid solution. It is removed immediately and placed in a dish containing running tap water. The final step is the flooding of the slide with methylene blue which is poured off after a few seconds, washed briefly with running tap water and dried with filter paper. Rickettsiae are stained a bright pink or red against a bluish background. This stain is especially good for the Psittacosis-Lymphogranuloma group.

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EXPERIENCES WITH THE ROUTINE EXAMINATION OF CORD SPECIMENS BY COOMBS TEST*

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The antihuman globulin test described by Coombs, Mourant, and Race in 1945[1] has been employed extensively through the intervening years in the examination of cord specimens for evidence of sensitization in hemolytic disease of the newborn. Its value has been mainly appreciated in detection of Rh sensitization. Recent reports[2,3] indicate that this technique may be applied as well in investigations in which antibodies other than Rh or Hr are involved. Of most importance is the appearance of positive Coombs tests in cases of ABO incompatibilities in which the mother is group O and the fetus group A or group B.

In most cases, Rh sensitization of the newborn can be predicted by showing antigenic differences between the mother and father and by demonstration of antibody titre in the maternal serum. In ABO sensitization, however, results of study of the maternal serum are at best equivocal due to the presence of natural-occurring anti-A and anti-B components. In this type of incompatibility the first born is frequently affected, though usually to a slight degree, in the absence of history of previous maternal sensitization[4].

This paper deals with a series of anti-human globulin tests performed routinely during a six months period for the obstetrical service. The work was not undertaken as a survey, but represents findings in a routine procedure performed on all specimens of cord blood collected within this period. The purpose of these tests was chiefly the detection of Rh sensitization in those cases in which prenatal investigation of the mothers as to Rh factor had not been done. One instance of ABO sensitization was demonstrated. Follow-up studies of these cases demonstrating positive Coombs results were not performed in the routine work-up as these are done only on special request by the attending physician.

Collection and Storage of Specimens

The specimens were collected at the time of delivery by the attending nurse. Tubes of uniform size were properly labeled with name of patient and room number. A requisition slip accompanied the specimen to the laboratory. No maternal history was received by the laboratory prior to the performance of the test. Specimens which were collected at night remained in the the delivery room station until they were brought to the laboratory in the morning. No refrigeration was employed prior to receipt in the laboratory. Upon arrival at the laboratory, specimens were immediately refrigerated until such time as the examinations could be conveniently carried out.

Handling Procedure

Specimens were tested daily excepting Sundays at 8:00 A.M. Specimens received after this time were refrigerated and tested the following morning. Cord bloods from known Rh negative mothers were marked "stat" when delivered to the laboratory and were tested immediately at any time —day, night, or week-end. The number of specimens tested in the daily routine ranged from six to twenty-four.

* Read before the 25th Anniversary Convention of ASMT, June 1957, Chicago, Illinois.
** Work done at St. Paul Hospital, Dallas, Texas.

Tests Employed

Cord bloods on all cases delivered in the hospital, regardless of history, were subjected to Rh factor determination and Coombs test. D^u determinations were not done on specimens tested Rh negative except in those cases in which atypical reactions were observed in the routine typing procedure. Further studies in cases of positive Coombs findings were left to the discretion of the attending physician. The Rh typing procedure consisted of testing with anti-D serum only. Genotypes were not determined routinely.

Methods

Rh Typing Tube method was employed in testing for the Rh factor. The rack was set up with two tubes (10 x 75 mm) for each specimen tested. Two drops of commercial anti-D serum were added to one tube, two drops of 22% albumin to the other, which served as the control tube. In preparing the clots, excess serum was removed and discarded. Two sticks were inserted and the clot was broken up to free the cells. One stick was placed in each tube of a pair for all specimens tested. The rack was then shaken and the sticks removed and discarded. The tubes were transferred to the centrifuge for one minute at 1,000 rpm. The results were then read macroscopically, the albumin tube being the negative control.

In cases of cord bloods, agglutination in the albumin tube may usually be attributed to the presence of Wharton's jelly in the specimen. Wharton's jelly is a mucilageneous substance present in the umbilical cord which may be accidentally introduced into the cord blood at the time of collection. These cases of agglutination may be eliminated by first washing the cells three times in saline before adding the albumin or anti-D serum. The washed cells are tested in the same manner as cells from whole blood.

D^u testing of all specimens typed Rh negative in the above procedures is highly desirable but was not possible in our studies. On occasions, bloods reacting questionably in the above procedures were tested and found to be D^u positive reactors. These cases have not been enumerated in this report because all cases were not examined for the presence of D^u.

Direct Coombs Test The cord blood rack was arranged with a tube (10 x 75 mm) for each specimen. A 2% suspension of each respective cord blood was prepared in its properly labeled tube. The cells were washed three times with tubesful of warm (37° C) saline. The saline was decanted completely following the last washing and two drops of commercial anti-human globulin (Coombs) serum added. The test was incubated for the period recommended by the producer of the serum and followed by centrifugalization at 1,000 rpm for one minute. The results were read macroscopically for agglutination. A positive Coombs test may indicate sensitization to Rh, Hr, A, B, or to other rarer factors. One of our cases was shown to be due to ABO incompatibility.

Positive Coombs results in those cases showing history compatible with Rh, Hr, or ABO iso-sensitization were frequently followed by genotype studies on mother, child, and father; antibody titre determination of the mother's serum and cross matching of suitable blood for possible exchange transfusion for the baby.

In cases of possible ABO incompatibility, grouping of mother, father, and child was done and immune anti-A or anti-B antibody titration of

mother's serum and elution tests on the baby's cells performed. These tests are not included in the routine procedure but are done only in cases properly evaluated by the attending physician.

Results

During the six months period, April through September, 1956, 2,166 cord blood specimens were examined as described. Of these, seven were found to be Coombs positive, usually of a four plus grade. Also among these, one apparently due to ABO incompatibility was encountered having a two plus direct Coombs reaction. These limited results compare with Davidsohn's incidences[4] predicted for random deliveries of one Rh incompatibility in three hundred and about one ABO incompatibility in three thousand.

Sources of Error

Several sources of error may have been effective in our results:

1. The inability to perform tests immediately after collection of specimens in all cases.
2. Inadequate refrigeration between time of collection and time of performance of tests.
3. Loss of antibody due to washing of cells with warm (37° C) saline.
4. Negative results in weak sensitizations due to prozone reactions.
5. Use of single commercial anti-serum not adequately checked in our own laboratory against known weakly reacting cells.
6. Missing of weak positive reactions by doing macroscopic readings only.
7. Lack of follow-up studies to stress importance of plus-minus reactions.
8. Inadequate time in a routine procedure to properly evaluate weakly positive or plus-minus reactions, although rechecks were done in doubtful cases whenever possible.
9. Some instances of use of personnel unaccustomed to careful search for weak positive or doubtful reactions.
10. Lack of maternal histories to alert technologists to the necessity for especially careful search for weak reactions in select cases.

Summary

Results of 2,166 routine cord blood specimens tested by direct Coombs methods have been reported. Routine testing of cord specimens, though yielding positive results in only a small percentage of cases, undoubtedly is of value in detecting cases of sensitization which would otherwise be overlooked. This procedure would, at least indirectly, aid in reducing infant mortality and morbidity. Routine Coombs testing of cord specimens would appear to be of value to those who have the time, personnel, and financial means to institute such a procedure. Sources of error, as listed, should be recognized and eliminated.

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A SIMPLIFIED TABLE FOR USE IN ANTISTREPTOLYSIN O DETERMINATIONS

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The purpose of this paper is to present a new, simplified table to be used in antistreptolysin O (ASTO) determinations. The advantages of the table are that it is simple to follow and that the positive control is included so that the ASTO titration and control can be more easily performed simultaneously.

The reagents used in our laboratory are purchased from Difco Laboratories, Detroit 1, Michigan and are reconstituted according to their directions.

Serum dilutions are made, 1:10, 1:100, and 1:500, as shown in the table using buffered solution. Test tubes, 15x85 mm., are labeled 1-19; tubes 1-12 are used for the ASTO titration, tubes 13-17 are the positive control tubes and tubes 18-19 are the red blood cell and streptolysin controls respectively.

ASTO TITRATION AND POSITIVE CONTROL TABLE

Tube No.	ml. of 1:10 serum dilution	ml. of 1:100 serum dilution	ml. of 1:500 serum dilution	Positive Control	Buffer	Todd Units
1	0.8	---	---	---	0.2	12
2	0.2	---	---	---	0.8	50
3	---	1.0	---	---	---	100
4	---	0.8	---	---	0.2	125
5	---	0.6	---	---	0.4	166
6	---	0.4	---	---	0.6	250
7	---	0.3	---	---	0.7	333
8	---	---	1.0	---	---	500
9	---	---	0.8	---	0.2	625
10	---	---	0.6	---	0.4	833
11	---	---	0.4	---	0.6	1250
12	---	---	0.2	---	0.8	2500
13	---	---	---	1.0	---	100
14	---	---	---	0.8	0.2	125
15	---	---	---	0.6	0.4	166
16	---	---	---	0.4	0.6	250
17	---	---	---	0.3	0.7	333
18	---	---	---	---	1.5	RBC Control
19	---	---	---	---	1.0	Streptolysin Control

The test can first be performed with two serum dilutions, or tubes 1-7, as a preliminary titration. It is preferable, however, to use three serum dilutions, or tubes 1-12, to avoid repeating the test if positive through two dilutions.

An ASTO titration and positive control table has been presented. The table is simple to follow and facilitates the performance of ASTO determinations.

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ABSTRACTS (Cont'd)

A MODIFIED CARBONATE-PHOSPHOTUNGSTATE METHOD FOR THE DETERMINATION OF URIC ACID AND COMPARISON WITH THE SPECTROPHOTOMETRIC URICASE METHOD

Richard J. Henry, et. al., (Bio-Science Laboratories, Los Angeles), Amer. Journal Clin. Path 28, 152-160 (1957).

This paper describes a phosphotungstate reduction method, using sodium carbonate without cyanide, in which Beer's law would be valid throughout a considerable range and in which turbidity would not occur using a Beckman model DU as well as the Klett filter photometer.

The results obtained by the alkaline phosphotungstate reaction seem to be identical whether cyanide is present or not. The only advantage in the use of cyanide seems to be that it provides increased sensitivity. The sensitivity of the carbonate method is proven by statistical analysis of the results obtained on fifty-five serum and thirty-nine urine specimans.

Lithium sulfate is added to the phosphotungstate acid reagent to prevent turbidity or crystalline precipitate during the color development stage.

MODIFIED BLENDER CUP FOR HOMOGENIZING SMALL TISSUE SAMPLES

N. B. Furlong, Jr., Wright Air Development Center, U. S. Air Force. Jan. 1957. 11 pages. (Order PB 131207 from OTS, U. S. Department of Commerce, Washington 25, D. C.., 50 cents.)

This report describes a device succesfully used at the Air Force School of Aviation Medicine for routine tissue analyses. The device adapts a standard tissue blender motor for homogenization of tissue samples weighing from one to 20 grams. Its use allows modification of mechanical homogenization techniques to small tissue samples which previously could be accomplished only with glass pestle homogenizers. The mechanical techniques save time and effort and result in a greater uniformity of blending. It was successfully used for macerating the tissues of the heart, liver, kidney, skeletal muscle, and lung.

A REVIEW OF THE CLINICAL ENTITY AGAMMAGLOBULINEMIA AND ITS LABORATORY DIAGNOSTIC METHODS*

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The descriptive term "agammaglobulinemia" was coined originally to denote a syndrome in patients in which one of the symptoms is a complete absence of plasma and extravascular gamma globulin. Recently the term has been extended by some investigators to include cases of hypoproteinemia. A superficial similarity exists in both conditions, that being a deficiency of gamma globulin.

Bruton[1] was apparently the first to report a complete case study of agammaglobulinemia. His patient was an 8 year old boy who, beginning at the age of 4 years, had 19 attacks of respiratory infections which were usually complicated by pneumonia. When there was no demonstrable antibody titer to autogenous pneumococcal vaccines on four occasions, and repeated injections of diphtheria toxoid failed to bring forth any anti-toxin response, it was reasonably assumed that the patient lacked the ability to synthesize antibodies. An electrophoretic analysis of the patient's serum revealed the complete absence of gamma globulin. When serum globulin was administered at monthly intervals, the patient remained free of infections without recourse to other prophylactic measures.

Since this report was published in 1952, numerous other cases have rapidly accumulated in the literature.[2-10] Bruton and associates[11] reported on two additional cases later in the year. Janeway et al[12] in the following year presented observations on nine cases of agammaglobulinemia. Spain and his coworkers[13] followed with three additional case reports.

Prior to these reports, other investigators had noticed patients with either a relative or complete deficiency of gamma globulin. Schick and Greenbaum[14] in 1945 described a case of a 12 year old girl whose plasma showed a complete absence of gamma globulin, but who did not appear to be unusually susceptible to bacterial disease. The authors attributed the apparent agammaglobulinemia to a congenital defect in protein anabolism.

Krebs[15] in 1946 reported on a hypogammaglobulinemic condition in a 15 year old girl. The low level of globulin was evidently due to hypoproteinemia resulting from a prolonged period of malnutrition. Administration of a high protein diet returned the gamma globulin to within normal range.

Many cases of hypoproteinemia are discussed in the literature.[16-20] A review of these cases along with reports of agammaglobulinemia shows that in both conditions, the common denominator is the lowering of the gamma globulin concentration. However, other aspects such as clinical histories and diagnostic features present an array of differences

* 2nd SPF Award, Serology, 25th Anniversary Convention ASMT, June 1957, Chicago, Illinois.

between the two categories, the dissimilarities possibly reflecting differing etiologies of the globulin deficiencies.

In hypoproteinemia a disturbance in protein metabolism results in a depression of all the plasma protein fractions. The depression may be due to a lack of proteinaceous intake, to faulty protein breakdown or synthesis, or to inordinately rapid destruction of serum protein. When the disorder is due to dietary insufficiency, the situation may be alleviated by a high protein diet.

Agammaglobulinemia on the other hand is due specifically to a faulty globulin anabolism which results in immunoglobulin not being synthesized, while the other serum components are being formed at a normal rate.

In some cases of hypogammaglobulinemia, the globulin deficiency may approach a level too low to be detected by electrophoretic tools. As pointed out by Fried and Henley,[20] even in cases which are apparently agammaglobulinemia insofar as electrophoretic analysis is concerned, the concentration of gamma globulin may be suffcient to protect the individual against infections. This is supported by the hypoproteinemic case of Schick and Greenbaum[14] where there was no increased susceptibility to infections in spite of the lack of gamma globulin. The negative Schick and Dick tests however, indicated the presence of some antibodies in this patient.

It has been pointed out that 100-150 milligrams of gamma globulin per 100 ml of serum is a sufficient quantity to prevent the occurrence of infectious processes,[11] but such values must be viewed with some reservations. One must also consider the factors of native resistance in total immunity when critical values of globulin in relation to immunity are discussed.

In hypoproteinemia, innate resistance appears to be lowered,[21] but whether this is also the case in agammaglobulinemia remains a question to be answered.

The diversity of conditions resulting in hypogammaglobulinemia, their clinical pictures and diagnostic features cannot be presented within the scope of this discussion. Agammaglobulinemia, being a single clinical entity, will be briefly reviewed within the limitation of this paper.

The disorder is not a generalized protein dysmetabolism, but rather, in Good and Varco's[22] words, an "immunologic handicap." Features which characterize the condition are: (a) an increased susceptibility to bacterial disease, (b) the absence of gamma globulin in the serum, (c) the absence of antibodies in the blood and tissues, and (d) the failure of antibody production in response to antigenic stimulus.

The Schick test is persistently negative, even following repeated injections of diphtheria toxoid. The Dick test likewise remains negative C-Reactive protein is formed in these individuals during the course of inflammatory and necrotizing processes.[24] The erythocyte sedimentation rate is also elevated; thus indicating that although agammaglobulinemic individuals lack the ability to form antibodies, they do retain the capacity to produce other altered serum constituents.

It should be noted that increased rate of protein catabolism is not a factor in these cases, as it has been found that the half life of injected

gamma globulin in these patients is the same as in normal individuals.[23]

Janeway[12] has suggested that congenial agammaglobulinemia is an inherited condition which is sex-linked to the male. Prasad and Koza[5] believe that in addition to the congenital form, these is also an acquired type of agammaglobulinemia to which both males and females are susceptible. This opinion was advanced on the basis of observations on the many cases of agammaglobulinemia in adults of both sexes, many of whom during their earlier years enjoyed relatively good health without abnormal disposition to bacterial infections.

The hematologic pictures in both the congenital and acquired forms are varied and discordant. Lymphopenia has been reported in some cases, but lymphocytosis has also been cited. However in cases where bone marrow studies and lymph node biopsies have been made, it has been consistently found that there is an absence or scarcity of plasma cells. Furthermore, there has been no evidence of plasmacytosis in response to antigenic injections.

Most other laboratory tests have not been of value for the diagnosis of the disorder. Liver function tests such as thymol turbidity and cephalin cholesterol flocculation have been negative. Total protein and albumin-globulin ratio are usually within normal limits. The zinc turbidity test for gamma globulin is considered useful for screening.[22] It remains, however, for electrophoretic studies and a few serologic and immunologic tests to provide us with the required information. Even with these tools however, the differentiation between congenital and acquired forms of agammaglobulinemia is a problem which remains to be solved.

Paper electrophoresis separation of serum is usually carried out in barbital buffer at a pH of 8.6. At this pH, the gamma globulin is stationary at the point of application, while the other components migrate toward the anode. The rate of migration is dependent upon the electrical charges of the various fractions, the net charges determining the mobility. Upon completion of the separation procedures, the paper is stained with bromphenol blue dye, this being sufficient for a qualitative evaluation of the serum. Quantitation of the fraction, if necessary, may be made with an automatic scanning device, or by the dye elution technique. The normal serum gamma globulin range is 13-16% of the total protein value.

The zinc turbidity test is based on the principle that a turbidity is formed when serum is treated with a buffered zinc solution. The turbidity is apparently related to the concentration of serum gamma globulin. The density of the turbidity is measured nephelometrically. Normal value range for this test is 2-12 Maclagen units.

Vaccine injections such as with typhoid or pertussis antigens, diphtheria or tetanus toxides, usually induce a reactive titer within a period of two to three weeks in a normal subject. A pre-injection and a 3 weeks post-injection serum sample can be used to assess the functional capability of the antibody forming organs.

Isoagglutinin determinations are easily performed with known group A and B cells. The usual procedure is to determine the patient's blood type, and then to titrate his serum against the appropriate type of cells.

In agammaglobulinemia the isoagglutinins are absent.

Along the same line, injections of incompatible blood or Witebsky blood group substances may be used to confirm the lack of antibody response.

In laboratories where electrophoresis equipment is not readily available, the absence or presence of gamma globulin may be detected by a hemagglutination-inhibition test as reported by Wiener.[25a,b] In principle the test relies on the agglutination of sensitized cells with anti-human globulin (Coombs') serum. Normal human serum containing gamma globulin inhibits such agglutination. Serum from agammaglobulinemic patients, on the other hand, permits the reaction to proceed because of the absence of the inhibiting fraction. The sensitivity of the test is inversely related to the titer of the anti-globulin serum used. According to Grubb,[26] as little as 0.1 gamma of gamma globulin may be detected by this technique.

A laboratory technique which can easily be adapted for screening use for agammaglobulinemia is the gel diffusion technique. A report of its use was made by Gell.[27] The test is performed by placing patient's serum and anti-globulin serum in cups cut in agar. The reactants diffuse from the cups, and a line is formed where precipitation occurs. In agammaglobulinemia no precipitation occurs. A word of caution must be injected at this point concerning the use of commercial anti-human globulin serum for this technique. For unknown reasons, absorbed preparations will not give this reaction. In our laboratory antiserum for this test is obtained by injections of gamma globulin into rabbits.

The author is fortunate in having been able to perform some laboratory studies on a globulin-treated case of agammaglobulinemia. Electrophoretic analysis, zinc turbidity, isoagglutinin titration, hemagglutination-inhibited and gel diffusion reactions were attempted. Because of the presence of gamma globulin remaining from therapeutic injections, the demonstration of the various techniques has not been as striking as would otherwise be the case.

The patient under study is a two year old male who had had numerous bouts with bacterial infections since birth. When electrophoretic studies of serum protein were made, the complete absence of gamma globulin was noted. Since the establishment of the diagnosis at age 1½ years, monthly injections of gamma globulin have been given. Susceptibility to infections has been conspicuously reduced. The blood sample used here for the demonstrations was taken just prior to the monthly injection.

A normal control was selected of the same sex, age, stature and blood type. Other controls were also used to assess the sensitivity and specificity of the tests. Included also was a patient with multiple myeloma whose serum in paper electrophoresis showed a large amount of globulin migrating slightly forward of the gamma globulin fraction in the control.

Electrophoresis. Separation of the fractions of the patient's and control sera was made with a model R Spinco paper electrophoresis apparatus. Quantitation was obtained with a model RA Spinco Analytrol Scanner. Interpretation of the tracings gave the following percent distribution of the protein components:

		Patient	Control
Albumin		59.7%	43.9%
Globulin:	alpha-1	5.2%	6.0%
	alpha-2	16.6%	19.7%
	beta	12.4%	16.0%
	gamma	6.2%	14.4%

Total protein determinations were performed on the sera by the Biuret method. Values obtained were 6.3 and 6.1 grams per 100 ml of serum for the patient and control sera respectively. In terms of absolute quantity of gamma globulin, the patient had 390 milligrams %, in comparison with 880 milligrams % of the control.

Zinc Turbidity. The test was performed by adding 0.1 ml of serum to 6 ml of zinc-barbital buffer. After standing at room temperature for 30 minutes, the turbidity was measured spectrophotometrically using a wave length of 650 mu. The patient's serum gave a value of 0.9 units, while the control serum was 8.5 units.

Hemagglutination-Inhibition. The Coombs' inhibition test performed was a modification of the technique reported by Grubb.[26]

Buffered saline was used throughout the test. Three volumes of 0.85% NaCl solution were added to one volume of M/15 phosphate buffer of pH 7.4. For the preparation of sensitized cells, pooled group O, Rh positive cells were washed three times and suspended to a 2% concentration. Sensitization was effected by mixing 1 ml of commercial anti-D serum containing blocking antibodies to 5 ml of cell suspension. The mixture was incubated at 37°C for one hour, followed by three washings. The cells were constituted to a 2% suspension. Commercial Coombs' serum was used for performance of the test. Optimal proportion titration was performed, 1:40 dilution of anti-globulin serum selected for use. The amount of anti-D serum used for sensitization of the cells was also determined by this titration.

Performance of the test: Serial dilutions of the patient's serum were made beginning with 1:100, the unit being 0.2 ml. To each tube was added 0.2 ml of Coombs' serum dilution. The tubes were shaken and incubated for 30 minutes at 37°C. Two-tenth (0.2) ml of sensitized cells was added to each tube, and the test left at room temperature for four hours. The reaction was graded on the basis of the pattern of cell deposit. Appropriate controls were included with the test. The results are tabulated in Table I.

It can be seen from the results that there is a four-fold dilution difference between the patient and the control titrations. The inhibition seen in the first four tubes of the patient's serum undoubtedly is due to the residual amount of gamma globulin remaining from previous injections.

Isoagglutinin Determination. The patient's blood type was found to be group A. Serial dilutions of his serum and the control serum were made in 0.5 ml units beginning with 1:2. To each tube was added 0.2 ml of 2% suspension of group B, Rh negative cells. The tubes were incubated for 15 minutes at room temperature and then lightly centrifuged for three minutes. The control showed agglutination in serum dilution of 1:512, while the patient's serum did not agglutinate the cells in 1:2 dilution. The results are presented in Table II.

TABLE I

Hemagglutination-Inhibition Test

	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	CONTROLS	
											Coombs	Serum
Patient Control	—	—	—	—	—	—	—	—	+	+	+	—
Patient	—	—	—	—	+	+	+	+	+	+		—
Precipitated Globulin	—	—	—	—	—	—	—	+	+	+		—
Human Albumin	+	+	+	+	+	+	+	+	+	+		±
Multiple Myeloma	—	—	—	+	+	+	+	+	+	+		—
Normal Control A	—	—	—	—	—	—	—	—	+	+		—
Normal Control B	—	—	—	—	—	—	—	—	+	+		—
Normal Control C	—	—	—	—	—	—	—	+	+	+		—

+—Hemagglutination Coombs' Control — Coombs' serum dilution + sensitized cells
Serum Control—1:100 dilution of serum + sensitized cells

TABLE II

Isoagglutinin Titration

	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	Saline Control
Patient	—	—	—	—	—	—	—	—	—	—	—	—
Control	+	+	+	+	+	+	+	+	+	—	—	

+ — Hemagglutination Saline Control — Saline + type B cells.

Gel Diffusion Reaction. The reaction was performed with 1% agar in saline buffered to pH 7.4. The agar was poured in a small petri dish to a depth of 8 mm. Cups 6 mm in diameter were obtained by means of a template. The arrangement of the cups was so that the central cup was surrounded by four cups at a radius of 10 mm. Anti-human globulin serum was obtained from rabbits injected with gamma globulin. The rabbit serum was placed in the center cup, and the antigen sera placed in the outer cups. The antigen sera used were from (a) the agammaglobulinemic patient, (b) the normal control, (c) a multiple myeloma patient and (d) a normal adult control.

The reaction was allowed to proceed at room temperature. Readings were taken at 24 hours, 72 hours and five days. Precipitation lines began to form within 24 hours. At 72 hours the lines were distinct. For both the agammaglobulinemic patient and the multiple myeloma patient, a faint line was observed. The two controls displayed distinct and heavy lines. The arrangement of the cups and the observed reactions are diagrammatically illustrated in Figure I.

Summary

Since the advent of antibiotics for the control of bacterial infections, the clinical entity of agammaglobulinemia has been brought to light. Antibodies which play a primary role in acquired immunity are totally lacking when the disorder is present. Naturally occurring blood group isoagglutinins are also absent. The impairment may be present at birth or may be acquired at a later age for reasons which are beyond our knowledge presently.

Laboratory techniques at hand for the diagnosis of the condition are electrophoresis, zinc turbidity, hemagglutination-inhibition, gel diffusion reaction and isoagglutinin determination. The principles and techniques of the various tests are reviewed.

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URINE PROTEIN TESTS IN PRESENCE OF TOLBUTAMIDE METABOLITE

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Treatment of diabetes with tolbutamide (ORINASE)[1] leads to the appearance in the urine of a metabolite which is acid insoluble. This metabolite (1-butyl-3-p-carboxyphenylsulfonylurea) is precipitated in common tests for urine protein such as the sulfosalicylic acid tests, the nitric acid ring test, and the heat and acetic acid test. This phenomenon causes false positive results when the urine of tolbutamide-treated patients is tested for protein by these methods.

Two new colorimetric tests for urine protein have been introduced recently.(1)(2) One is a tablet test called ALBUTEST[2] and the other is a dip-test called ALBUSTIX.[3] These tests both involve the same principles and depend on the color change of an indicator in the presence of protein. The present report describes comparison of these tests with common turbidimetric tests for urine protein in a small series of urines containing tolbutamide metabolite along with study of the reactivity of the various tests with purified metabolite added to normal urine.

Table I shows the results of tests applied to a series of ten urine samples from patients or healthy subjects receiving tolbutamide. It will be observed that these urines gave strongly positive turbidity tests in all instances. Only one urine gave a strongly positive reaction with the color tests and two other urines gave trace reactions with the color tests.

TABLE I

Protein Tests on Urines Containing Tolbutamide Metabolite

Type of Test	Number of Urines Showing Result Indicated		
	Negative	Trace	Strongly Positive
Sulfosalicylic Acid Test	0	0	10
Nitric Acid Ring Test	0	1	9
Albutest	7	2*	1*
Albustix	7	2*	1*

* Specimens demonstrated to contain protein.

Further study of these three urines indicated that when they were dialyzed against distilled water the material responsible for the color reaction did not pass through a cellophane membrane, thus suggesting that these three urines did contain protein. The further identity of protein in the sample which was strongly positive was confirmed by biuret-reactivity in the material precipitated with sulfosalicylic acid after dialysis. In contrast, experiments indicated that the metabolite of tolbutamide which precipitates in the urine protein turbidity tests does pass through a cellophane membrane on dialysis.

Isolation of tolbutamide metabolite from the urine of a subject ingesting tolbutamide was accomplished by solvent extraction with 1:1 ether-butanol. This material was recrystallized from methanol and identified by neutral equivalent and nitrogen analysis. Protein-free urine samples were saturated with metabolite by stirring with an excess for 5 minutes and

[1] ORINASE is a trademark of Upjohn Co. Inc., Kalamazoo, Mich.
[2] ALBUTEST is a registered trademark of the Ames Company Inc., Elkhart, Ind.
[3] ALBUSTIX is a trademark of Ames Company Inc., Elkhart, Ind.

decanting the clear supernatant. The urines with purified metabolite added were then tested with the turbidimetric sulfosalicylic acid test, the nitric acid ring test, the colorimetric tablet test and the colorimetric dip test. In every instance the sulfosalicylic acid test and the nitric acid ring test were strongly positive whereas the colorimetric tablet test and the colorimetric dip-test were completely negative.

These results suggest that the simple colorimetric tablet test and dip-test for proteinuria are convenient means of recognizing protein in urine containing tolbutamide metabolite since such urines give false positive results with the common turbidity tests.

A clinical study by Dr. A. A. Silver[3] in diabetics receiving tolbutamide has indicated that the color tests are negative in urines showing pseudo-albuminuria with the sulfosalicylic acid test.

SUMMARY

Tolbutamide metabolite which causes false positive reactions with common turbidity tests for protein does not react with the new colorimetric tablet test (ALBUTEST) or dip-test (ALBUSTIX) for proteinuria. These colorimetric tests do readily react with protein in the presence of tolbutamide metabolite.

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MEDICAL TECHNOLOGISTS NEEDED

(Continued, see Page xxx)

26th Annual Convention of the American Society of Medical Technologists

Milwaukee, Wisconsin—June 15-20, 1958

I LIKE MILWAUKEE

By CHARLES HOUSE

I like Milwaukee

I like it because it sleeps at night the way a well behaved city should . . . I like it as it is: conservative, hesitant, provincial, but keenly anxious for growth and improvement . . .

I like it because it polishes its own shoes and wears its hat square and true without the rakish, affected angle . . .

* * *

I like the foolish, homely streets which were built on Indian trails more than a hundred years ago . . . I like the glowering beetle-browed buildings which have somehow lived on into a new era . . . I like the sweeping, swinging dignified beauty of Lake Drive.

I like the way our city nestles against the blue waters of Lake Michigan . . and I enjoy the gentle liquid sound of its waves patting a caress on our shore when the day is kind . . . and I like the bold, whistling wind as it thunders off the lake in a storm.

* * *

I like Milwaukee because it is big . . . I like it because it is little, too, for it is the only city in America which is both . . . I like it because it has selected a mayor who is gentle and able, and who rides home on a street car like the people he serves . . . I like Milwaukee's big, hulking red-faced policemen who are friendly, honest men . . . I like to watch the gyrations of the barrel chested traffic officer who is on stage daily at 3rd and Wells Streets, and I like to hear the eloquence of his whistles, which have brought him some local fame . . .

I like the slanted, dejected buildings which are askew, humble and unpainted on the side of the river, but which are prim and bright on the North Water Street front . . . I like the humble dignity of our little homes and the slender grandeur of our inadequate city hall which was built for a lesser city than today's Milwaukee . .

I like the crisp twang in the speech of Milwaukeeans . . . and I admire the still-present red-cheeked hausfrau and her ample husband, who is the undisputed head of the household . . . and will be always . . .

I like Milwaukee because the world admires our safety records, our health records, our efficient police and fire departments, and wonders enviously how we do it . .

* * *

I like my city because ladies still bake wonderful-smelling pies and handsome cakes, which they share with the neighbors as they did long, long ago . . . I like Milwaukee because neighborliness is a force here though it is long since dead in many another city of equal size . . . I like the way folks helped each other during the great storm two years ago . . . and I remember with warm pleasure how many people showed an eager willingness to help stranded strangers when public transportation failed . . .

* * *

I like the quiet streets where the house lights go out at 10:30 p.m. and where the kids are still taught to say their prayers . . . I like the down-to-earth righteousness of Milwaukee and the uneasiness which buzzes through the city when a rare crime is committed . . . I like Milwaukee's simplicity and sincerity . . .

* * *

I like the plans Milwaukee makes for tomorrow and the earnestness with which each projected plan is wrought, somehow, into final shape after good, hard, healthy wrangling back and forth between factions . . . I like our respect for the past and our hope for the future . . .

I like Milwaukee.

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Downtown Milwaukee

Milwaukee's Harbor

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